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Integrated multivariate approach of ecological and ecotoxicological parameters in coastal environmental monitoring studies

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ABSTRACT

The coastal ecosystems are recognized worldwide by their ecological an economic importance. In these ecosystems, aquatic life is governed by the abiotic variations, therefore, the interactions between natural stressors and anthropogenic pollution (multiple stressors) may represent an additional threat. Thus, considering these combined effects with the climate change scenarios, it is urgent to develop cost-effective tools to assess the impact of pollution on sensitive and important ecosystems, such as estuaries and coastal lagoons, and implement mitigation plans to prevent the environmental and human health degradation. In order to contribute to this crucial issue, the central objective of this study was to validate and propose an integrated methodology, including ecological and ecotoxicological parameters, to evaluate the effects of natural stressors and pollution on key estuarine fish in real scenarios, using the common goby *Pomatoschistus microps* (Krøyer, 1838), an important estuarine intermediary predator, as a bioindicator and a North Atlantic coastal lagoon (the Aveiro lagoon; NW coast of Portugal) as a case study area. The methodology included fourteen water quality variables, sediment characteristics, the concentrations of nine metals in sediments and in the fish body, fish condition indexes, eight biomarkers and multivariate statistics (Redundancy (RDA) and Principal Response Curves analysis (PRC)) to integrate the information provided by different parameters. The study was conducted over one year with seasonal sampling (winter, spring, summer and autumn) at four sampling sites with different contamination histories. The integrated approach suggested significant differences between the reference site and the remaining ones, both in biological and environmental descriptors. Bioaccumulation factors suggested that fish bioaccumulated some metals, especially Zn. The overall integration of all selected parameters and methods discriminated locations with different contaminated levels and associated health status of the selected species with multiple stressors. The present work represented a case-study, highlighting the use of biomarkers as early warning indicators of ecosystems health and stressing the importance of using an integration approach through multivariate analysis in biomonitoring studies, providing an important baseline and integrated information that may be used as scientific support for conservation and management and ecological models of estuarine and coastal lagoon systems.

1. Introduction

Coastal transition ecosystems, such as estuaries and coastal lagoons, are among the most productive and valuable aquatic ecosystems on earth. They are recognized worldwide as an important component of continental coasts in terms of their biological importance and human utilization (Marques et al., 2004), being crucial to the life history and development of many species (Chapman and Wang, 2001; Mahoney and Bishop, 2017). A considerable part of these areas around the world have been increasingly contaminated by chemicals resulting from anthropogenic activities. In this context, metals are pollutants of concern. They are known to accumulate in sediments and in organisms, being toxic at ecological relevant concentrations (Kennish and Paerl, 2010; Van Der Oost et al., 2003). These contaminants may also alter water and sediments characteristics, decreasing their overall quality to support the community of organisms living in their dependency (de Souza

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Machado et al., 2016). In fact, despite the essential role of some metals, several of those, commonly found in estuaries and lagoons, are able to disrupt several physiological functions of organisms (Frasco et al., 2007; Vieira et al., 2009) with potential effects at higher levels of biological organization. Furthermore, since some species occupying low trophic levels are able to accumulate metals (Battuello et al., 2016; Cairrão et al., 2007), predators of these species may be exposed to toxic doses even if biomagnification does not occur. Therefore, monitoring programs have been carried out to assess the type and levels of metals in sediments and water (e.g. Ajmone-Marsan et al., 2008; Coelho et al., 2014; Vicente-Martorell et al., 2009), and the bioaccumulation of these pollutants by selected species, especially those for human consumption (Chi et al., 2007; Pereira et al., 2011). However, chemical analyses *per se* are not sufficient to describe the adverse effects of these pollutants (Ozmen et al., 2006). In these ecosystems, considerable variations of physic-chemical parameters use to occur and several species are already living in their tolerance limits relatively to some of these factors. Thus, the interactions between natural stressors (e.g. temperature, salinity, pH and light) and metals remain a complex issue to be clarified and may be an additional threat at least to some species. Therefore, considering global changing scenarios, it is urgent to develop cost-effective methods to assess the impact of anthropogenic pollution, including metals, on sensitive and important ecosystems such as estuaries and coastal lagoons. Particular attention should be given to field approaches assessing both direct toxic effects on organisms and alterations on the abiotic component of the ecosystem.

Among animals inhabiting coastal lagoons, fish are of great interest since distinct species may occupy different ecological niches, they are sensitive to several environmental contaminants and some species have economic importance. Therefore, several fish have been used as sentinel species in monitoring programs carried out in estuaries, lagoons and coastal areas (Cabral et al., 2007; Capela et al., 2016; Guimarães et al., 2009; Rodrigues et al., 2006; Solé et al., 2006; Webb et al., 2005). One of this species is the common goby, *Pomatoschistus microps* Krøyer (1838), which is an abundant fish in estuaries, coastal lagoons and shores of Europe (Arruda et al., 1993; Salgado et al., 2004) where it plays an important ecological function as intermediary predator connecting macro- and meiofauna with larger predator fish (Arruda et al., 1993; Miller, 1986).

In the last years, several monitoring programs with fish included environmental biomarkers to assess biological adverse effects of chemicals present in water, sediments and/or fish body (Capela et al., 2016; Monteiro et al., 2007). These parameters allow the early diagnosis of effects resulting from stress exposure, long before they become evident at the population level where adverse effects may be difficult to revert. Enzymes involved in physiological processes determinant for the survival and performance of organisms, as well as markers of oxidative damage, are among the most used biomarkers in biomonitoring studies. Some examples are: acetylcholinesterase (AChE) which plays a determinant role in cholinergic neurotransmission; lactate dehydrogenase (LDH) which is a key enzyme in the anaerobic pathway of energy production; glutathione S-transferases (GST) that are involved in detoxification and in lipid peroxidation prevention; catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) which are part of the anti-oxidant defences, and lipid peroxidation (LPO) that has been used as a marker of oxidative damage (Barhoumi et al., 2014; Falfushynska et al., 2009; Ferreira et al., 2008; Guimarães et al., 2009; Monteiro et al., 2007; Solé et al., 2006). These biomarkers used simultaneously with health condition indexes, such as the Fulton Condition Factor (FCF) and the Hepatosomatic Index (HSI), determined in representative samples and in relation to a reference population, allow a good indication of the general health *status* of the population in relation to chemical exposure and its effects.

Despite the urgent need to validate cost-effective tools to assess the combined impact of natural stressors and anthropogenic pollution on coastal ecosystems, a significant lack of knowledge and methodology integration, still exists. These studies, including the implementation of assessment systems in monitoring programmes, the reference conditions, typologies and intercalibration are now a priority in marine/estuarine ecotoxicology and extremely important to implement mitigation plans to prevent the environmental and human health degradation. To contribute to these challenges, the central objective of this study was to validate and propose an integrated methodology, including ecological and ecotoxicological parameters, to evaluate the effects of natural stressors and metal pollution on key estuarine fish in real scenarios, using an important estuarine intermediary predator (*P. microps*), as a bioindicator and a North Atlantic coastal lagoon (the Aveiro lagoon; NW coast of Portugal) as a case study area. Recent studies on climate changes have highlighted the high sensitivity of South European marine coastal environment to climate variability (IPCC, 2014; Vieira et al., 2015), reinforcing the need of integrated multivariate biomonitoring studies in this area. The approach included twenty physico-chemical parameters indicative of water quality and sediment characteristics, the concentrations of nine metals (Al, Pb, Cd, Cr, Co, Cu, Ni, Zn and Hg) in sediments and in the fish body, eight biomarkers determined in different tissues of fish, two health condition indexes and multivariate analysis to integrate the information provided by different parameters.

2. Material and methods

2.1. Chemicals

The chemicals for analysis were purchased from Sigma–Aldrich® Chemical (Steinheim, Germany), except the Bradford reagent (Bio-Rad®, Munich, Germany). The chemicals used for water analysis were from Palintest® (Palintest LTD – England).

2.2. Short description of the study area and sampling sites

The Aveiro lagoon is located in the NW coast of Portugal (Fig. 1). It is approximately 45 km long and 10 km wide, covering a minimum area of approximately 66 km^2 at low spring tide, and reaching a maximum of 83 km^2 at a high spring tide (Dias et al., 2001). This lagoon is supplied with freshwater by two main rivers: the Antuã river (5 $m^3 s^{-1}$ average flow) and the Vouga river (50 $m^3 s^{-1}$) (Dias et al., 1999). It is a very irregular and complex coastal lagoon, composed by long and narrow channels, with a high longitudinal development organized by successive ramifications from the mouth, as an arborescent network system (Leandro et al., 2007; Morgado et al., 2003). This lagoon is composed of a wide range of biotopes (e.g. wetlands, salt marshes and mudflats) used as nursery areas for many valuable species belonging to different animal groups, such as bivalves, crustaceans, fish and birds (Lopes et al., 2007).

The study was performed during one-year (2005–2006), with seasonal sampling (winter, spring, summer and autumn). This ecosystem has been subjected to anthropogenic pressure through waste loadings, some of which with high metal content (Pires et al., 2017; Velez et al., 2015). For this reason, four sites were selected reflecting different degrees of anthropogenic contamination by metals (Fig. 1). A global position system (GPS) (Garmin GPSMAP 60CSX) was used to determine the coordinates of the sampling sites (Fig. 1), which are briefly described below:

- *Barra* (40°37′50.91″N, 8°44′38.96″W) is located near to the artificial connection of lagoon to the sea. This sampling site was chosen as reference site due to its low levels of environmental contamination (Cerqueira and Pio, 1999; Quintaneiro et al., 2006; Lillebø et al., 2015) and because it has been used as a reference site in a previous study with *P. microps* (Monteiro et al., 2007) and other species (Guilherme et al., 2008; Quintaneiro et al., 2006; Nilin et al., 2012).
- *Vagueira* (40°34′24.32″N, 8°45′20.60W) is located in the Mira channel of the Aveiro lagoon which receives agriculture and animal

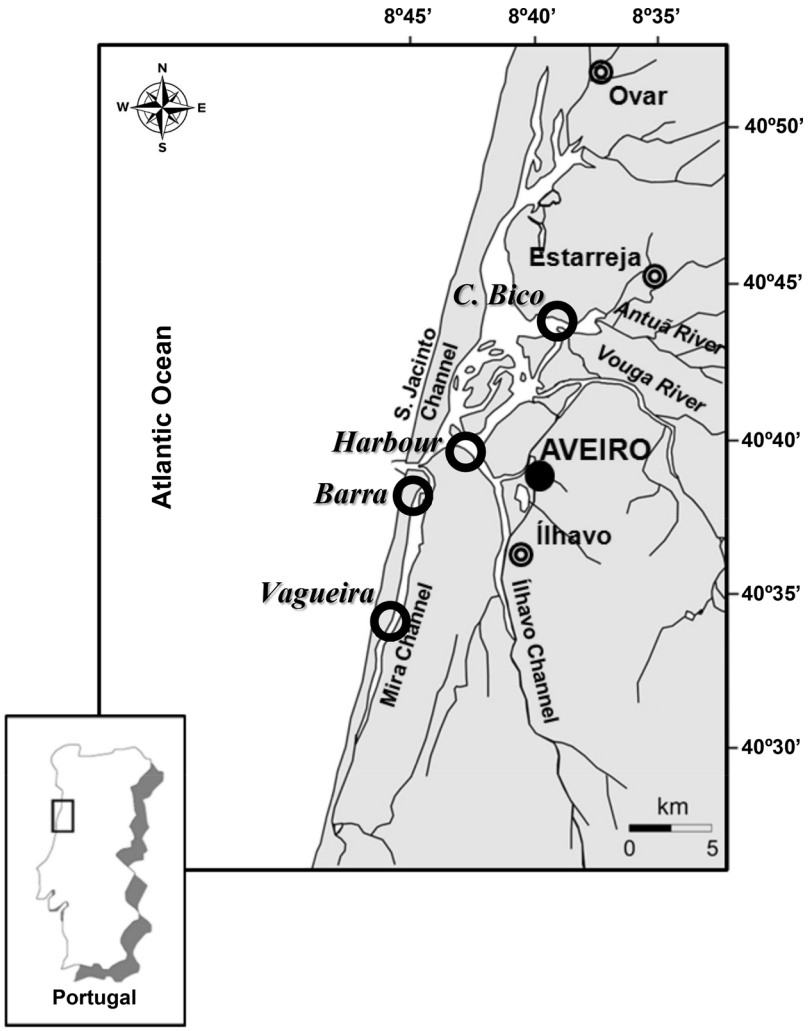

Fig. 1. The Aveiro lagoon indicating the location of the selected sampling sites, main channels and Rivers: Barra (40º37′50.91″N, 8º44′38.96″W), Vagueira (40º34′24.32″N, 8º45′20.60W), Harbour (40º39′19.56″N, 8º42′13.00″W) and C. Bico (40º43′46.96″N 8º39′00.13″W).

farming run-off and, therefore, it is likely to be contaminated by fertilizers and pesticides used in crop fields and additives used for animal growth. Significant levels of metals in the Mira Channel (in southern areas from this site) had been reported in other studies (Duarte et al., 2017; Fonseca et al., 2014, 2015; Nunes et al., 2008)

- *Harbour* (40°39′19.56″N, 8°42′13.00″W) is located in the Aveiro harbour. It is contaminated by petrochemical products and their components, including metals (Hall et al., 1987; Martins et al., 2013; Pacheco et al., 2005).
- *Cais do Bico* (*C. Bico*) (40°43′46.96″N; 8°39′00.13″W) is located near an area known as Laranjo bay which is described as polluted by metals (Fonseca et al., 2015; Monterroso et al., 2003a, 2007; Nilin et al., 2012).

2.3. Fish and sediments sampling

P. microps juveniles (2.5–3 cm long) were captured seasonally at the four selected sites using a hand operated net at low tide. Approximately 105 animals were collected at each site for morphometric parameters, condition indexes and biomarkers (Section 2.5) and 360 individuals for determination of the concentrations of metals. After being collected, animals were immediately transported to the laboratory in thermally isolated and aerated boxes. In the laboratory, fish were weighted, measured and sacrificed by decapitation. Then, the eviscerated body weight was also determined. All the weight determinations were done using an analytical balance (KERN 770). Liver was isolated on ice and individually weighted using the same analytical balance. Two condition indexes were determined: the Fulton Condition Factor (FCF) and the Hepatosomatic Index (HIS) as:

$$FCF = 100 \times \frac{W}{L^3}$$

Where W is the total fish weight (g) and L is the total body length (cm) (Pyle et al., 2005).

$$HIS = \frac{l}{b} \times 100$$

Where l is the weight of liver (g) and b is the total body weight (g) (Lloret et al., 2002).

Sediment samples for particle size determination (granulometry (GR)), organic matter content (OM), chlorophyll *a*, *b* and *c*, and phaeopigments assays were collected seasonally (winter to autumn) at the same time of fish, at the four selected sites (Fig. 1). For each parameter, 10 replicates from the first 1 cm of sediment were collected per site, with a 100 ml syringe (previously washed with nitric acid). In addition, sediments for metals chemical analyses (section 2.6) were collected (in triplicate) at each sampling location, from the top layer (1-cm depth) as indicated previously. All sediment samples collected were stored in plastic bags, commonly used for keeping human food, previously washed with nitric acid. Once in the laboratory, the samples were stored at −20 °C until analysis. Granulometry determination involved a splitting process of the sediment into a sand fraction (particles greater than 63 μm) and a silt-clay fraction (SCF) (particles less than 63 μm), following the methodology described by Holme and McIntyre (1971). The organic matter content was estimated after combustion of

previously dried samples, following the methodology indicated by Strickland and Parsons (1972). For chlorophylls and phaeopigments determinations, each sediment replicate was added to a 50 ml polyethylene centrifuge tube with 30 ml of 90% acetone and 0.2 ml of magnesium carbonate (1 g/100 ml). At each sampling site, the centrifuge tubes were mixed thoroughly and, in the lab, they were kept in the fridge for 24 h until the assay. Chlorophyll *a*, *b* and *c* and phaeopigments were estimated spectrophotometrically following the methodology described in Strickland and Parsons (1972), with small adaptations.

2.4. Water variables

Water analyses were performed in samples collected seasonally, including at the time of fish and sediment sampling. Temperature (°C), salinity (‰), conductivity (μS/m) and pH were measured *in situ* at the four sampling sites during low tide with a multi-parameter probe (Hydrolab DS5X–Hach Environmental). Dissolved oxygen (DO) (%) was measured *in situ* using a portable meter (LDO HQ10–Hach Environmental). At each sampling site, subsurface water samples were also collected to 0.5 l polyethylene-terephthalate bottles and stored at −20 °C for analysis. Levels of nitrates ($mg\ NO_3\ L^{-1}$), nitrites ($mg\ NO_2\ L^{-1}$), ammonia ($mg\ NH_4\ L^{-1}$), phosphates ($mg\ PO_4\ L^{-1}$), silica ($mg\ Si\ L^{-1}$), phenol ($mg\ C_6H_5OH\ L^{-1}$), iron, hardness ($mg\ CaCO_3\ L^{-1}$) and turbidity (FTU) were measured using commercial photometer kits (Photometer 7000, Palintest, Kingsway, England).

2.5. Biomarkers determination

From each fish, head, gills, dorsal muscle and liver were isolated on ice. The heads of 15 fish were put in 4.5 ml of phosphate buffer (0.1 M, pH 7.2) and used to prepare one sample for AChE determinations; pieces of dorsal muscle from 15 different fish were put in 4.5 ml of Tris-NaCl buffer (0.1 M, pH 7.2) and used to prepare one sample for LDH determinations; 15 pairs of gills were put in 4.5 ml of phosphate buffer (0.1 M, pH 6.5) and used to prepare one sample for GST analysis and 15 livers were put in 1:10 (w/v) of phosphate buffer (0.05 M, pH 7.0, with 0.1% Triton X-100) and used to prepare samples for LPO and antioxidant enzymes. Seven replicate pooled samples were prepared per site and per biomarker, in a total of 105 fish per site. Samples were homogenised on ice using an Ystral homogenizer (Ballrechten-Dottingen, Germany) and centrifuged (SIGMA 3 K 30 centrifuge) as follows: for 3 min at 4 °C and 3300*g* for AChE and LDH samples; for 30 min at 4 °C 9000 g for GST determinations. After homogenization of AChE, LDH and GST samples, the supernatant of each homogenate was collected and stored at −80 °C until further analysis. In the case of homogenised liver, 200 μl were collected from each sample and then stored at −80 °C after addition of 4 μl of butylated hydroxytoluene (BHT). The remanding homogenised liver of each pooled sample was centrifuged (SIGMA 3 K 30 centrifuge) for 15 min at 15000*g* (4 °C). Each of the final supernatants was divided in aliquots for CAT, SOD, GR and GPx assays and stored at −80 °C. Prior to enzymatic analysis, the protein of each sample was normalised to $0.3\ mg\ ml^{-1}$ in the case of samples for AChE and GST determinations, and to $0.9\ mg\ ml^{-1}$ in the case of samples for LDH, CAT, SOD, GR and GPx determinations (Vieira et al., 2008, 2009). The protein content was determined by the Bradford method (Bradford, 1976) adapted to microplate as indicated in Guilhermino et al. (1996).

AChE activity was determined according to the Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996), using acetylthiocholine as substrate and a microplate reader BIO-TEK, model POWERWAVE 340. In a previous study, it was found that the soluble fraction of *P. microps* head homogenates contains mainly acetylcholinesterase (AChE) (Monteiro et al., 2005). LDH was determined by the method of Vassault (1983), adapted to microplate (Diamantino et al., 2001), using a microplate reader BIO-TEK, model POWERWAVE 340. GST was assessed according to Habig et al. (1974), with the modifications of the original protocol indicated in Frasco and Guilhermino (2002), in a microplate reader BIO-TEK, model POWERWAVE 340. The activities of GR, GPx and SOD were determined according to the methods of Carlberg and Mannervik (1975), Flohé and Günzler (1984) and McCord and Fridovich (1969), respectively, adapted to microplate (Lima et al., 2007). A microplate reader BIO-TEK, model POWERWAVE 340 was used. CAT activity was measured according to the method of Aebi (1984) in a spectrophotometer JENWAY, model 6405 UV/VIS. LPO levels were determined by the quantification of thiobarbituric acid reactive substances (TBARS) as in Ohkawa et al. (1979) and expressed as nmol of TBARS per g of tissue, using a spectrophotometer JENWAY, model 6405 UV/VIS. At the end of enzymatic biomarkers and LPO analysis, the amount of protein in each sample was again determined and this value was used for expressing the activity.

All the enzymatic activities were determined at 25 °C and expressed as activity per mg of protein. One unit (U) of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%. For CAT activity, one U was defined as 1 μmol/min and for all the remaining enzymes one U was equivalent to 1 nmol/min.

2.6. Chemical analysis and bioaccumulation factors

The following metals were measured in sediments and in the fish body: Al, Pb, Cd, Cr, Co, Cu, Ni, Zn and Hg. Sediment samples for chemical analysis were collected in the field and transported to the laboratory as previously indicated (Section 2.3). In the laboratory, the sediments were dried in a drying oven (Raypa®) at 40 °C, they were put in previously nitric acid washed plastic bags, covered with aluminium foil and sent for Terracon Laboratorium (Jüterbog, Germany) where the determination of heavy metals content was done. Here, 2 g of homogenised sediment was weighed into a 250 ml reaction glass vessel (accuracy 0.1 mg); 5 ml of nitric acid (68%, trace select quality) and 15 ml of hydrochloric acid (37%, trace select quality) were added ("aqua regia" acid digestion). The reaction vessel with cooler and vapour trap was heated at 180 °C for 2 h and the acidic solution was filled to 50 ml in a volumetric flask with 5% of nitric acid solution. After filtration, the metals were measured by ICP-OES. An aliquot of the sediment sample was used to determine the dry matter (mT) at 105 °C. Metals were determined with ICP-OES (Model ARL 3410) after calibration with external standards for each element. Mercury (Hg) was determined with Hydrid-AAS (Model UNICAM 939) after calibration with an external standard.

Fish collected in the field for chemical analysis were weighted in an analytical balance (KERN 770). Then, groups of 120 individuals (1 replicate) were put in 15 ml Falcon tubes, previously washed with nitric acid, protected from light with aluminium foil, and kept at −20 °C until analysis. Three replicates were prepared per site and season. Samples were lyophilised (freeze drying) for 4 days in a EZ-DRY lyophilizer (model n° EZ55OQ − FTS Systems, USA) and packed in Falcon tubes previously washed with nitric acid, protected from light with aluminium foil. In the laboratory of chemical analyses, 0.5–0.8 g of the fish sample were weighed (accuracy 0.1 mg), put into a 150 ml glass reaction vessel and 5 ml of sulphuric acid (98%, trace select quality) were added. After reaction overnight ("wet washing" with sulphuric acid to destroy the organic part of the sample), 1 ml of nitric acid and 1 ml of hydrogen peroxide were added. The vessel with a vapour trap was heated in a microwave digestion apparatus according to a temperature program up to 200 °C for 45 min. If the solution was not clear after the first digestion, further 1 ml of nitric acid and 1 ml of hydrogen peroxide were added and a new microwave digestion was started. These steps were repeated until a clear and colourless solution was obtained ("total acidic microwave digestion"). The digestion was carried out with the original air-dried (max. 40 °C) fish sample. The acidic solution was

Table 1
Results from Repeated Measures Analysis of Variance (RM-ANOVA), for environmental variables measured both in water and sediment, using sampling site as factor. The Dunnett's Comparisons results represents the post hoc comparisons with the reference site (Barra), performed using the Dunnett's Simultaneous Test. Season was selected as a random factor; therefore, no multiple comparisons were performed. Temp – temperature, Cond – conductivity, Sal – salinity, DO – dissolved oxygen, NO_3 – nitrates, NO_2 – nitrites, NH_4 – ammonia, PO_4 – phosphates, SiO_2 – silica, C_6H_5OH – phenol, Fe-w – iron (in water), Hard – hardness, Turb – turbidity, Chl *a* – Chlorophyll *a*, Chl *b* – Chlorophyll *b*, Chl *c* – Chlorophyll *c*, Phaeo – phaeopigments, SCF – Silt Clay Fraction and OM – organic matter.

Water	Parameter	Factor	Statistics	Dunnett's Comparisons (*)
	Temp	site	$F_{(3,41)} = 7.44$ ($p < 0.001$)	***Barra*** *Harbour C. Bico Vagueira*
		season	$F_{(3,41)} = 539.58$ ($p < 0.001$)	
	Cond	site	$F_{(3,41)} = 7.52$ ($p < 0.001$)	*C. Bico Vagueira Harbour* ***Barra***
		season	$F_{(3,41)} = 35.39$ ($p < 0.001$)	
	Sal	site	$F_{(3,41)} = 12.37$ ($p < 0.001$)	*C. Bico Vagueira Harbour* ***Barra***
		season	$F_{(3,41)} = 120.17$ ($p < 0.001$)	
	pH	site	$F_{(3,41)} = 4.24$ ($p < 0.05$)	Vagueira *C. Bico* ***Barra*** *Harbour*
		season	$F_{(3,41)} = 14.49$ ($p < 0.001$)	
	DO	site	$F_{(3,41)} = 3.99$ ($p < 0.05$)	*Harbour Vagueira* ***Barra*** *C. Bico*
		season	$F_{(3,41)} = 49.60$ ($p < 0.001$)	
	NO_3	site	$F_{(3,41)} = 18.74$ ($p < 0.001$)	***Barra*** *Harbour Vagueira C. Bico*
		season	$F_{(3,41)} = 31.66$ ($p < 0.001$)	
	NO_2	site	$F_{(3,41)} = 3.45$ ($p < 0.05$)	***Barra*** *C. Bico Harbour Vagueira*
		season	$F_{(3,41)} = 6.10$ ($p < 0.05$)	
	NH_4	site	$F_{(3,41)} = 11.26$ ($p < 0.001$)	***Barra*** *C. Bico Harbour Vagueira*
		season	$F_{(3,41)} = 15.62$ ($p < 0.001$)	
	PO_4	site	$F_{(3,41)} = 4.92$ ($p < 0.05$)	***Barra*** *Harbour C. Bico Vagueira*
		season	$F_{(3,41)} = 8.20$ ($p < 0.001$)	
	SiO_2	site	$F_{(3,41)} = 11.51$ ($p < 0.001$)	***Barra*** *C. Bico Vagueira Harbour*
		season	$F_{(3,41)} = 64.23$ ($p < 0.001$)	
	C_6H_5OH	site	$F_{(3,41)} = 5.35$ ($p < 0.05$)	***Barra*** *C. Bico Harbour Vagueira*
		season	$F_{(3,41)} = 14.34$ ($p < 0.001$)	
	Fe-w	site	$F_{(3,41)} = 8.46$ ($p < 0.001$)	***Barra*** *Vagueira Harbour C. Bico*
		season	$F_{(3,41)} = 21.44$ ($p < 0.001$)	
	Hard	site	$F_{(3,41)} = 22.13$ ($p < 0.001$)	*C. Bico Vagueira Harbour* ***Barra***
		season	$F_{(3,41)} = 38.45$ ($p < 0.001$)	
	Turb	site	$F_{(3,41)} = 11.53$ ($p < 0.001$)	***Barra*** *Vagueira Harbour C. Bico*
		season	$F_{(3,41)} = 18.16$ ($p < 0.001$)	
Sediment				
	Chl *a*	site	$F_{(3,153)} = 27.91$ ($p < 0.001$)	*C. Bico* ***Barra*** *Harbour Vagueira*
		season	$F_{(3,153)} = 22.24$ ($p < 0.001$)	
	Chl *b*	site	$F_{(3,153)} = 11.97$ ($p < 0.001$)	*C. Bico Harbour* ***Barra*** *Vagueira*
		season	$F_{(3,153)} = 28.28$ ($p < 0.001$)	
	Chl *c*	site	$F_{(3,153)} = 12.10$ ($p < 0.001$)	*C. Bico* ***Barra*** *Harbour Vagueira*
		season	$F_{(3,153)} = 17.00$ ($p < 0.001$)	
	Phaeo	site	$F_{(3,153)} = 17.88$ ($p < 0.001$)	*Harbour C. Bico* ***Barra*** *Vagueira*
		season	$F_{(3,153)} = 38.98$ ($p < 0.001$)	
	SCF	site	$F_{(3,153)} = 12.59$ ($p < 0.001$)	*Harbour Vagueira C. Bico* ***Barra***
		season	$F_{(3,153)} = 14.59$ ($p < 0.001$)	
	OM	site	$F_{(3,153)} = 30.92$ ($p < 0.001$)	***Barra*** *Harbour Vagueira C. Bico*
		season	$F_{(3,153)} = 4.66$ ($p < 0.05$)	

(*) Sites statistically not different from the reference are united by an underscore.

filled to 50 ml in a volumetric flask with 5% nitric acid solution. After filtration, the metals were measured by ICP-OES (Model ARL 3410) after calibration with external standards for each element. Mercury (Hg) was determined with Hydrid-AAS (Model UNICAM 939) after calibration with an external standard.

The bioaccumulation factor (BAF) was calculated for each metal according to the following formula (Barron, 1995):

$$BAF = \frac{Cfish}{Csed}$$

Where, *Cfish* is the concentration (mg/kg) of metal in the fish tissue and *Csed* is the concentration (mg/kg) of the same metal in the sediment. For each metal and site, the C*sed* was the mean of the concentrations individually determined for the three samples of sediments collected at that site; the BAF was calculated for each fish as the ratio between the concentration of metal in that fish and the mean of sediment samples concentration.

2.7. Statistical analyses

Variables were tested for normality (Anderson-Darling normality test) and homogeneity of variance (Levine's test) (Zar, 1996). Strong departures from normality and homoscedasticity were corrected with log(x), log (x + 1), arcsin[sqrt(x)] or sqrt(x) transformations, as appropriate. The values of each environmental variable, each metal determination, each condition index and each biomarker were compared over time by Repeated Measures Analysis of Variance (RM-ANOVA) using sampling site as factor. When statistically significant differences among sites were found, post hoc comparisons were done against "*Barra*" using the Dunnett's test (Zar, 1996). Season was selected as a random factor; therefore, no multiple comparisons were performed for this factor. The null hypothesis of no differences between the reference and contaminated sites was tested, except in the case of metals in sediments where the null hypothesis was that contaminant levels in the sediment from the contaminated sites were less or equal to the levels of the reference site.

In addition, the ordination technique of redundancy analysis (RDA), an ordination method of direct gradient analysis (Ter Braak and Prentice, 1988) was done to assess the relationship between biological and environmental data. The biomarkers, condition indexes and metals analysed in fish were selected as species data (later on referred as biological descriptors), water parameters and chemical analysis of

metals in sediments were selected as environmental data (later on referred as environmental descriptors) and environmental parameters in sediment were selected as covariables data. Monte Carlo permutations were used to assess statistical significance of the canonical axes. Moreover, the least significant environmental variables, after Monte Carlo permutations under reduced model, were automatically excluded from the graphical representation.

The biological and environmental data were also analysed by Principal Response Curves (PRC). PRC is based on RDA, the constrained form of Principal Component Analysis (Van den Brink and ter Braak, 1999). The analysis resulted in diagrams showing the seasons on the x-axis and the first Principal Component of parameters on sites, on the y-axis. This yield a diagram showing the deviations in time of the most contaminated sites in relation to the reference (*Barra*). The weights of analysed parameters, biological descriptors (biomarkers, condition indexes and metals analysed in fish) and environmental descriptors (metals analysed in sediment and environmental parameters measured in water) are presented in separate diagrams indicating the affinity of the parameters with responses displayed in the first diagram.

Statistical analyses were performed using the software Minitab 14.0® (Minitab Inc, USA), with the exception of RDA and PRC analyses that were done using the software CANOCO 4.5© for Windows (Biometris, The Netherlands) (ter Braak and Smilauer, 1998).

3. Results

3.1. Characterization and comparison of sampling sites

The mean, maximum and minimum values of the parameters measured in water and sediments are described in the Table A1 in Appendix A. The results from RM-ANOVA for environmental variables measured both in water and sediment are shown in Table 1. Significant differences among seasons and sites were found for all the tested parameters in both water and sediments (Table 1). *Vagueira* showed significant differences relatively to the reference site (*Barra*) in the following parameters: temperature, conductivity, salinity, pH, nitrates, nitrites, ammonia phosphates, silica, phenol, hardness, turbidity, sediment chlorophyll *a* and *c*, and sediment organic matter. *Harbour* showed significant differences relatively to *Barra* in water nitrates, nitrites, ammonia, phosphates, silica, iron, hardness and turbidity, and sediment chlorophyll *c*, phaeopigments and silt clay fraction. *C. Bico* showed significant differences relatively to *Barra* in water conductivity, salinity, nitrates, phosphates, silica, iron, hardness and turbidity, and sediment chlorophyll *a* and *b*, and organic matter. It should be noted that all the contaminated sites had higher values of nitrates and phosphates than *Barra*, while *Vagueira* and *Harbour* also had higher concentrations of nitrites and ammonia than *Barra*.

The mean concentrations of the nine metals determined in the sediments collected seasonally at the four sampling sites are shown in Table A2 in Appendix A. Cd was only found in one season (winter) and in one site (*Vagueira*) and thus it was not included in the statistical analysis. Significant differences among sites and seasons were found for all the metals, with the exception of Pb variation among seasons and Cu among sites (Table 2). Relatively to the reference site, *Vagueira* showed significantly higher concentrations of Al, Cr, Co and Ni. *C. Bico* showed statistically significant higher concentrations of Al, Pb, Co, Zn and Hg relatively to *Barra*, while no statistically significant differences between *Harbour* and *Barra* were found for any of the metals determined in the sediments.

3.2. Bioconcentration of metals

The concentrations of the nine metals determined in *P. microps* collected from the four sampling sites in different seasons are shown in the Annex A.3. and the results of statistical analysis are indicated in Table 3.

Statistically significant differences among sites were found for Al, Cd, Zn and Hg. Relatively to fish from the reference site, animals from *C. Bico* showed significantly higher levels of Zn and Hg, fish from *Vagueira* showed significantly lower concentrations of Al, while those collected at *Harbour* had significantly lower concentrations of Al and higher levels of Zn (Tables 3 and A3 in Appendix A). The BAFs calculated for each metal are displayed in Table 4. and the results of RM-ANOVA analysis are

Table 2
Results from RM-ANOVA statistical analysis, for the nine metals measured in sediment, using sampling site as factor. The Dunnett's Comparisons results represents the post hoc comparisons with the reference site (shown in bold), performed using the Dunnett's Simultaneous Test. Season was selected as a random factor; therefore, no multiple comparisons were performed. NS = not significant at $p<0.05$.

Parameter(*)	Factor	Statistics	Dunnett's Comparisons (**)
Al	site	$F_{(3,41)} = 16.73$ ($p<0.001$)	*Harbour* ***Barra*** *C. Bico Vagueira*
	season	$F_{(3,41)} = 4.05$ ($p<0.05$)	
Pb	site	$F_{(3,41)} = 7.97$ ($p<0.001$)	***Barra*** *Harbour Vagueira C. Bico*
	season	$F_{(3,41)} = 0.30$ (NS)	
Cr	site	$F_{(3,41)} = 10.05$ ($p<0.001$)	*Harbour* ***Barra*** *C. Bico Vagueira*
	season	$F_{(3,41)} = 6.89$ ($p<0.05$)	
Co	site	$F_{(3,41)} = 13.60$ ($p<0.001$)	*Harbour* ***Barra*** *C. Bico Vagueira*
	season	$F_{(3,41)} = 7.40$ ($p<0.001$)	
Cu	site	$F_{(3,41)} = 2.43$ (NS)	*Harbour* ***Barra*** *Vagueira C. Bico*
	season	$F_{(3,41)} = 6.83$ ($p<0.05$)	
Ni	site	$F_{(3,41)} = 5.76$ ($p<0.05$)	*Harbour* ***Barra*** *C. Bico Vagueira*
	season	$F_{(3,41)} = 6.09$ ($p<0.05$)	
Zn	site	$F_{(3,41)} = 17.31$ ($p<0.001$)	*Harbour* ***Barra*** *Vagueira C. Bico*
	season	$F_{(3,41)} = 4.94$ ($p<0.05$)	
Hg	site	$F_{(3,41)} = 11.70$ ($p<0.001$)	***Barra*** *Vagueira Harbour C. Bico*
	season	$F_{(3,41)} = 28.51$ ($p<0.001$)	

(*) Cd had only one record, in sediment, during the year (winter), therefore was not included in ANOVA analysis.
(**) Sites united by an underscore correspond to the situation where the null hypothesis (H_o: $\mu_{contaminated} \leq \mu_{reference}$) was not rejected.

Table 3
Results from RM-ANOVA statistical analysis, for the nine metals measured in *P. microps* tissues, using sampling site as factor. The Dunnett's Comparisons results represents the post hoc comparisons with the reference site (shown in bold), performed using the Dunnett's Simultaneous Test. Season was selected as a random factor; therefore, no multiple comparisons were performed. NS = not significant at $p<0.05$.

Parameter(*)	Factor	Statistics	Dunnett's Comparisons (*)
Al	site	$F_{(3,41)} = 4.45$ ($p<0.05$)	*Harbour Vagueira C. Bico* ***Barra***
	season	$F_{(3,41)} = 34.50$ ($p<0.001$)	
Pb	site	$F_{(3,41)} = 0.46$ (NS)	*Harbour* ***Barra*** *C. Bico Vagueira*
	season	$F_{(3,41)} = 3.58$ ($p<0.05$)	
Cd	site	$F_{(3,41)} = 4.88$ ($p<0.05$)	*C. Bico Harbour* ***Barra*** *Vagueira*
	season	$F_{(3,41)} = 11.37$ ($p<0.001$)	
Cr	site	$F_{(3,41)} = 0.12$ (NS)	*C. Bico* ***Barra*** *Vagueira Harbour*
	season	$F_{(3,41)} = 74.60$ ($p<0.001$)	
Co	site	$F_{(3,41)} = 0.11$ (NS)	*C. Bico Vagueira Harbour* ***Barra***
	season	$F_{(3,41)} = 30.51$ ($p<0.001$)	
Cu	site	$F_{(3,41)} = 0.11$ (NS)	***Barra*** *Harbour C. Bico Vagueira*
	season	$F_{(3,41)} = 2.69$ (NS)	
Ni	site	$F_{(3,41)} = 2.00$ (NS)	*Harbour Vagueira* ***Barra*** *C. Bico*
	season	$F_{(3,41)} = 0.63$ (NS)	
Zn	site	$F_{(3,41)} = 8.40$ ($p<0.001$)	***Barra*** *Vagueira C. Bico Harbour*
	season	$F_{(3,41)} = 39.18$ ($p<0.001$)	
Hg	site	$F_{(3,41)} = 7.85$ ($p<0.001$)	*Harbour Vagueira* ***Barra*** *C. Bico*
	season	$F_{(3,41)} = 21.04$ ($p<0.001$)	

(*) Sites statistically not different from the reference are united by an underscore.

Table 4
Annual bioaccumulation Factor (BAF) values for *Barra*, *Vagueira*, *Harbour* and *C. Bico*, for each metal, based in sediment and fish data. Values represent the mean ± S.E.M. The Cd concentrations were not included in BAF results.

BAF	Barra	Vagueira	Harbour	C. Bico
Al	**0.196** ± 0.056	**0.045** ± 0.013	**0.140** ± 0.041	**0.113** ± 0.024
Pb	**0.776** ± 0.224	**0.709** ± 0.204	**0.562** ± 0.162	**0.484** ± 0.139
Cr	**1.085** ± 0.313	**0.484** ± 0.135	**1.844** ± 0.535	**0.180** ± 0.052
Co	**0.493** ± 0.142	**0.766** ± 0.221	**3.664** ± 1.058	**0.375** ± 1.08
Cu	**1.622** ± 0.468	**3.530** ± 1.019	**4.068** ± 1.174	**2.956** ± 0.853
Ni	**3.067** ± 0.885	**1.086** ± 0.313	**0.619** ± 0.179	**1.386** ± 0.401
Zn	**8.001** ± 2.309	**6.761** ± 1.860	**20.96** ± 6.052	**6.069** ± 1.240
Hg	**2.678** ± 0.802	**3.417** ± 0.986	**2.436** ± 0.703	**2.925** ± 0.547

indicated in Table 5. Since Cd concentrations in sediments were detected only once, it was not possible to calculate the BAFs for this metal. BAFs higher than 1 were found for Cu, Zn Hg at all the sampling sites, for Cr at *Barra* and *Harbour*, for Co at *Harbour*, for Ni at *Barra*, *Vagueira* and *C. Bico*. The highest BAFs were found for Zn reaching 20.96. Significant differences in BAFs among sites and seasons were found for all the metals, except for Pb and Hg among sites, and Cu among sites and seasons. Statistically significant lower BAFs relatively to the reference site were found for Al and Ni in fish from *Vagueira*, and higher BAFs relatively to *Barra* were found for Co and Zn in fish from the *Harbour*.

3.3. Condition indexes and biomarkers in fish

The variation of *P. microps* condition indexes and biomarkers captured at different seasons and sampling sites is shown in Table 6. and the results of statistical analysis are shown in Table 7. Significant differences in FCF and HSI were found among sampling sites and seasons (Table 7). Statistically significant higher HSI were found in fish collected at all the contaminated sites relatively to those from the reference site. Despite significant differences in FCF among fish from different sites were also found, the Dunnett's Simultaneous Test was not able to discriminate the different groups of fish (Table 7).

Statistically significant differences among sites and seasons were found for all the tested biomarkers (Table 7). For all the biomarkers, the contaminated sites were significantly different from the reference site. Relatively to the fish collected at *Barra*, those from *Vagueira*, *Harbour* and *C. Bico* showed significantly inhibition of AChE (24%, 25%, 27%, respectively; % of inhibition calculated from annual means) and of LDH activities (Table 6). Increased LPO levels and activities of GST and antioxidant enzymes were found in fish from *Vagueira*, *Harbour* and *C. Bico* relatively to those from *Barra* (Table 6).

3.4. Integrated data analysis

The results of RDA are shown as a triplot ordination diagram (Fig. 2). The first two axes of the RDA analysis accounted for 62.6% (53.1% for biological descriptors and additional 9.5% for the interaction between biological and environmental data) of the overall canonical variability of the data. The first RDA axis (horizontal) accounted for 43.6% of the total canonical variability and was strongly associated with a seasonality gradient. Considering biological descriptors, this axis was particularly associated with Al (negative part) and Pb (positive part) concentrations in fish, while regarding environmental descriptors it was associated with Hg (negative part) and with temperature, iron, silica, turbidity, ammonia, phenol, pH, Al, Pb, Co, Cr, and Zn (positive part). The second constrained axis (vertical) accounted for additional 9% of the canonical variability and was influenced by contamination since the reference site (*Barra*) appears separated from the contaminated sites, especially in autumn and summer. The positive part of the axis is particularly associated with AChE, LDH and Ni (in fish) and conductivity, salinity and hardness (in water). The negative part of axis is mainly associated with LPO, HSI, antioxidant enzymes and Cu concentrations in fish, with nitrites, nitrates and phosphates in water and Cu in sediments.

The PRC diagrams displayed in Figs. 3 and 4. showed seasonal differences between *Barra* and the contaminated sites, considering the environmental and biological descriptors, respectively. Fig. 3. shows the PRC for the first and second axis (PRC1 and PRC2), resulting from the analysis of environmental descriptors (water parameters and metals in the sediment). Seasonal changes, sampling sites and replication accounted for 32.5%, 62.5% and 5% of the variance, respectively. The PRC1 (Fig. 3A) accounted for 48.6% of total variance. Differences between contaminated sites and *Barra* were found to be mainly associated

Table 5
Results from RM-ANOVA statistical analysis, for each metal BAF, using sampling site as factor. The Dunnett's Comparisons results indicate the post hoc comparisons with the reference site (control – shown in bold), performed using the Dunnett's Simultaneous Test. Season was selected as a random factor; therefore, no multiple comparisons were performed. NS = not significant at $p<0.05$.

Parameter (*)	Factor	Statistics	Dunnett's Comparisons (**)
Al	site	$F_{(3,41)} = 3.20$ ($p<0.05$)	*Vagueira* C. *Bico Harbour* ***Barra***
	season	$F_{(3,41)} = 8.71$ ($p<0.001$)	
Pb	site	$F_{(3,41)} = 0.21$ (NS)	*C. Bico Harbour Vagueira* ***Barra***
	season	$F_{(3,41)} = 3.38$ ($p<0.05$)	
Cr	site	$F_{(3,41)} = 7.25$ ($p<0.05$)	*C. Bico Vagueira* ***Barra*** *Harbour*
	season	$F_{(3,41)} = 4.67$ ($p<0.05$)	
Co	site	$F_{(3,41)} = 11.33$ ($p<0.001$)	*C. Bico* ***Barra*** *Vagueira Harbour*
	season	$F_{(3,41)} = 13.26$ ($p<0.001$)	
Cu	site	$F_{(3,41)} = 2.07$ (NS)	***Barra*** *C. Bico Vagueira Harbour*
	season	$F_{(3,41)} = 1.80$ (NS)	
Ni	site	$F_{(3,41)} = 3.96$ ($p<0.05$)	*Harbour Vagueira C. Bico* ***Barra***
	season	$F_{(3,41)} = 7.50$ ($p<0.001$)	
Zn	site	$F_{(3,41)} = 18.07$ ($p<0.001$)	*C. Bico Vagueira* ***Barra*** *Harbour*
	season	$F_{(3,41)} = 5.90$ ($p<0.05$)	
Hg	site	$F_{(3,41)} = 0.72$ (NS)	*Harbour* ***Barra*** *C. Bico Vagueira*
	season	$F_{(3,41)} = 3.14$ ($p<0.05$)	

(*) The Cd concentrations were not included in ANOVA analysis.
(**) Sites statistically not different from the reference are united by an underscore.

Table 6
Local and seasonal variation of condition indexes and biomarkers measured in *P. microps*, collected at the four sampling sites located in the Aveiro lagoon. FCF – Fulton Condition Factor and HSI – Hepatosomatic Index. The mean enzymatic activity, LPO and condition values, per year, for each location are shown in the grey column. Values indicate the mean ± S.E.M. U/mg protein = 1 μmol/min for CAT activity, the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% for SOD activity and 1 nmol/min for the other enzymes. AChE – acetylcholinesterase, LDH – lactate dehydrogenase, GST – glutathione S-transferases, CAT – catalase, SOD – superoxide dismutase, GR – glutathione reductase, GPx – glutathione peroxidase and LPO – lipid peroxidation.

	Barra					*Vagueira*					*Harbour*					*C. Bico*				
Condition Indexes	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean
FCF	**1.032** ± 0.045	**0.994** ± 0.021	**0.783** ± 0.025	**0.803** ± 0.052	**0.904** ± 0.027	**1.003** ± 0.071	**0.875** ± 0.012	**0.768** ± 0.033	**0.704** ± 0.076	**0.838** ± 0.033	**1.279** ± 0.049	**1.034** ± 0.032	**0.773** ± 0.042	**0.725** ± 0.042	**0.953** ± 0.047	**0.973** ± 0.027	**0.832** ± 0.026	**0.704** ± 0.033	**0.805** ± 0.072	**0.829** ± 0.027
HSI	**4.371** ± 0.373	**4.401** ± 0.235	**4.461** ± 0.175	**3.638** ± 0.181	**4.217** ± 0.136	**5.917** ± 0.202	**5.943** ± 0.216	**5.671** ± 0.255	**4.582** ± 0.361	**5.529** ± 0.165	**5.942** ± 0.252	**5.020** ± 0.251	**5.475** ± 0.201	**5.071** ± 0.151	**5.378** ± 0.125	**7.192** ± 0.355	**5.473** ± 0.066	**5.207** ± 0.154	**5.488** ± 0.231	**5.841** ± 0.185
Biomarkers																				
AChE (U/mg protein)	**39.97** ± 1.235	**31.12** ± 1.355	**44.67** ± 2.028	**27.82** ± 1.105	**35.90** ± 1.471	**27.65** ± 2.608	**30.72** ± 1.641	**35.95** ± 0.972	**14.95** ± 0.763	**27.32** ± 1.681	**29.82** ± 2.045	**26.99** ± 1.298	**34.51** ± 1.277	**15.88** ± 0.758	**26.81** ± 1.479	**31.61** ± 1.598	**27.01** ± 0.884	**31.81** ± 1.105	**14.05** ± 0.762	**26.12** ± 1.489
LDH (U/mg protein)	**252.4** ± 5.741	**171.4** ± 6.584	**244.4** ± 6.482	**205.1** ± 4.325	**218.3** ± 6.832	**219.1** ± 4.662	**142.7** ± 1.763	**235.7** ± 4.009	**104.7** ± 3.276	**175.6** ± 10.50	**189.2** ± 4.225	**131.9** ± 3.365	**161.2** ± 6.426	**145.9** ± 4.741	**157.1** ± 4.679	**173.6** ± 4.446	**154.8** ± 7.799	**186.7** ± 5.821	**121.6** ± 3.774	**159.2** ± 5.415
GST (U/mg protein)	**59.82** ± 0.798	**59.07** ± 3.156	**48.11** ± 1.068	**35.57** ± 1.626	**50.65** ± 2.097	**90.11** ± 3.572	**65.24** ± 2.636	**54.75** ± 2.273	**48.74** ± 2.757	**64.71** ± 3.325	**108.8** ± 2.605	**99.22** ± 2.058	**69.36** ± 1.477	**53.85** ± 2.091	**82.82** ± 4.373	**118.8** ± 2.076	**86.35** ± 3.913	**52.73** ± 2.609	**59.32** ± 2.718	**79.32** ± 5.203
CAT (U/mg protein)	**6.292** ± 0.321	**3.175** ± 0.253	**4.743** ± 0.453	**6.679** ± 0.247	**5.223** ± 0.308	**9.322** ± 0.445	**8.302** ± 0.468	**7.044** ± 0.571	**13.35** ± 0.202	**9.506** ± 0.501	**9.362** ± 0.218	**8.816** ± 0.471	**8.482** ± 0.386	**11.33** ± 0.256	**9.499** ± 0.268	**11.35** ± 0.321	**7.303** ± 0.437	**6.881** ± 0.524	**10.13** ± 0.375	**8.919** ± 0.413
SOD (U/mg protein)	**0.494** ± 0.019	**1.167** ± 0.134	**1.075** ± 0.025	**0.658** ± 0.017	**0.849** ± 0.631	**0.992** ± 0.021	**3.364** ± 0.156	**2.052** ± 0.059	**2.144** ± 0.075	**2.138** ± 0.167	**1.216** ± 0.055	**2.346** ± 0.121	**1.526** ± 0.077	**1.251** ± 0.048	**1.585** ± 0.095	**1.323** ± 0.036	**2.529** ± 0.041	**1.338** ± 0.024	**0.911** ± 0.027	**1.526** ± 0.117
GR (U/mg protein)	**2.853** ± 0.129	**2.261** ± 0.047	**5.686** ± 0.311	**4.752** ± 0.309	**3.888** ± 0.288	**6.627** ± 0.332	**3.893** ± 0.054	**9.578** ± 0.511	**11.19** ± 0.456	**7.824** ± 0.567	**7.269** ± 0.351	**4.148** ± 0.045	**8.580** ± 0.252	**10.16** ± 0.214	**7.542** ± 0.440	**9.679** ± 0.301	**5.216** ± 0.062	**7.277** ± 0.411	**9.967** ± 0.246	**8.035** ± 0.395
GPx (U/mg protein)	**7.017** ± 0.362	**5.351** ± 0.287	**14.56** ± 0.837	**5.957** ± 0.362	**8.222** ± 0.753	**14.08** ± 0.157	**14.09** ± 0.372	**32.16** ± 0.846	**19.17** ± 1.250	**19.88** ± 1.469	**17.61** ± 0.329	**16.82** ± 0.515	**17.87** ± 0.484	**15.45** ± 0.572	**16.94** ± 0.291	**16.02** ± 0.586	**10.44** ± 0.376	**30.41** ± 0.737	**17.35** ± 0.403	**18.56** ± 1.431
LPO (nmol TBARS/g tissue)	**88.46** ± 1.513	**90.51** ± 3.101	**47.25** ± 1.822	**40.38** ± 2.012	**66.65** ± 4.541	**185.9** ± 2.248	**176.6** ± 2.197	**65.64** ± 2.116	**135.1** ± 1.663	**140.9** ± 9.183	**122.8** ± 2.567	**126.1** ± 1.098	**62.36** ± 1.445	**91.21** ± 1.917	**100.6** ± 5.068	**171.5** ± 2.179	**158.7** ± 2.815	**79.67** ± 3.246	**124.4** ± 2.044	**133.6** ± 6.957

with Al, Zn and Cr concentrations in sediments (Fig. 3A). PRC2 analysis (Fig. 3B) accounted for 22.9% of total variance. For this second axis, the differences observed between contaminated sites and reference were found to be mainly associated with turbidity. Fig. 4. describes the PRC for the first and second axis (PRC1 and PRC2) resulting from the analysis of biological descriptors of *P. microps* (biomarkers, condition indexes and metals). Seasonal changes, sampling sites and replication accounted for 47.8%, 34.9% and 17.3% of the variance, respectively. The PRC1 and PRC2 accounted for 37.8% and 22.3% of total variance, respectively. Considering PRC1 analysis (Fig. 4A), the descriptors mainly responsible for deviations of *Vagueira*, *Harbour* and *C. Bico* relatively to the reference site were GPx, Pb, GR and CAT. For the PRC2 analysis (Fig. 4B) the differences observed between contaminated sites and *Barra* were found to be mainly associated with Ni and Pb levels in *P. microps* body.

4. Discussion

4.1. Characterization and comparison of sampling sites

The characterization of water quality is determinant for monitoring studies since it influence the life history and development of estuarine species, speciation, bioavailability and accumulation processes, and the organisms' responses to contamination levels (Bebianno et al., 2007;

Table 7
RM-ANOVA statistical analysis results for measured condition indexes and biomarkers in *P. microps*, using sampling site as factor. The Dunnett's Comparisons results indicate the post hoc comparisons with the reference site (shown in bold), performed using the Dunnett's Simultaneous Test. Season was selected as a random factor; therefore, no multiple comparisons were performed. For each biomarker and condition index, the full names are shown in Table 6.

Condition Indexes	Parameter	Factor	Statistics	Dunnett's Comparisons (*)
	FCF	site	$F_{(3,105)} = 5.66$ ($p < 0.05$)	*C. Bico Vagueira* ***Barra*** *Harbour*
		season	$F_{(3,105)} = 37.99$ ($p < 0.001$)	
	HSI	site	$F_{(3,105)} = 27.56$ ($p < 0.001$)	***Barra*** *Harbour Vagueira C. Bico*
		season	$F_{(3,105)} = 12.39$ ($p < 0.001$)	
Biomakers (**)				
	AChE	site	$F_{(3,105)} = 33.60$ ($p < 0.001$)	*C. Bico Harbour Vagueira* ***Barra***
		season	$F_{(3,105)} = 99.29$ ($p < 0.001$)	
	LDH	site	$F_{(3,105)} = 43.66$ ($p < 0.001$)	*Harbour C. Bico Vagueira* ***Barra***
		season	$F_{(3,105)} = 66.38$ ($p < 0.001$)	
	GST	site	$F_{(3,105)} = 56.88$ ($p < 0.001$)	***Barra*** *Vagueira C. Bico Harbour*
		season	$F_{(3,105)} = 110.32$ ($p < 0.001$)	
	CAT	site	$F_{(3,105)} = 66.29$ ($p < 0.001$)	***Barra*** *C. Bico Harbour Vagueira*
		season	$F_{(3,105)} = 47.72$ ($p < 0.001$)	
	SOD	site	$F_{(3,105)} = 97.17$ ($p < 0.001$)	***Barra*** *C. Bico Harbour Vagueira*
		season	$F_{(3,105)} = 88.44$ ($p < 0.001$)	
	GR	site	$F_{(3,105)} = 93.52$ ($p < 0.001$)	***Barra*** *Harbour Vagueira C. Bico*
		season	$F_{(3,105)} = 107.31$ ($p < 0.001$)	
	GPx	site	$F_{(3,105)} = 129.20$ ($p < 0.001$)	***Barra*** *Harbour C. Bico Vagueira*
		season	$F_{(3,105)} = 71.27$ ($p < 0.001$)	
	LPO	site	$F_{(3,105)} = 167.78$ ($p < 0.001$)	***Barra*** *Harbour C. Bico Vagueira*
		season	$F_{(3,105)} = 198.36$ ($p < 0.001$)	

(*) Sites statistically not different from the reference are united by an underscore.
(**) In post hoc comparisons all sites were different from the reference.

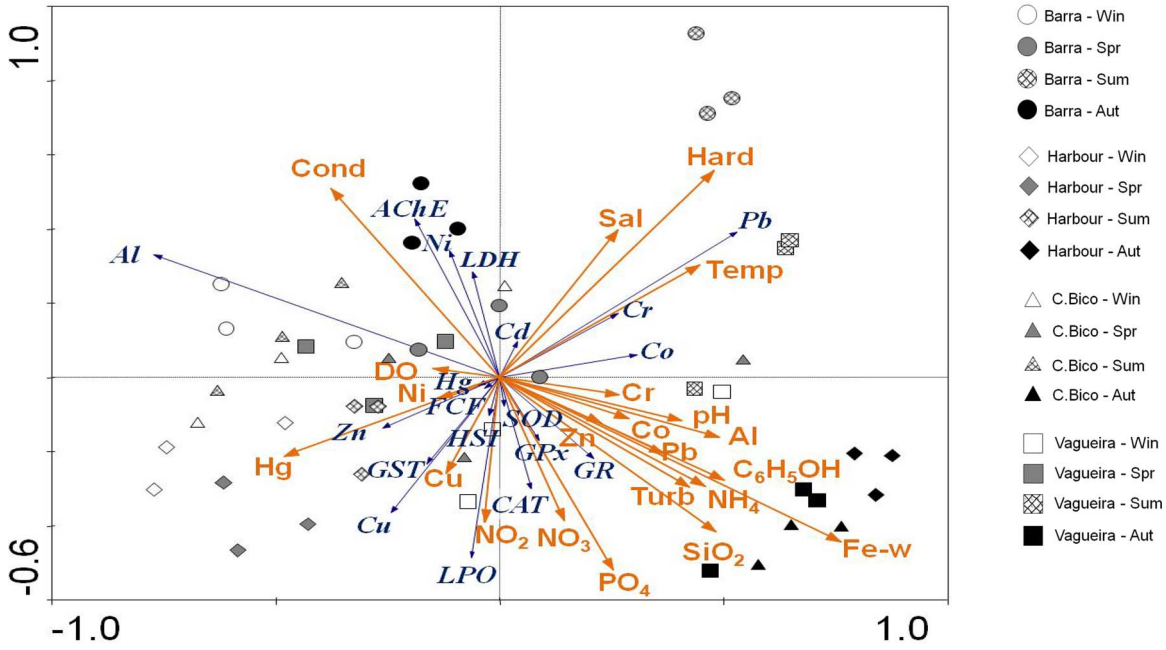

Fig. 2. Redundancy analysis (RDA) ordination diagram with biological and environmental data: the biomarkers, condition indexes and metals analysed in fish were selected as biological descriptors (blue), while water parameters and chemical analysis of metals in sediments were selected as environmental descriptors (orange). Environmental parameters analysed in sediment were selected as covariables data. The sampling sites are indicated as: circles – *Barra*; squares – *Vagueira*; rhombus – *Harbour* and triangles – *C. Bico*). For each sampling site is, also, indicated the season: Win – winter; Spr – spring; Sum – summer and Aut – autumn. First axis is horizontal; second axis is vertical. Temp – temperature, Cond – conductivity, Sal – salinity, Turb – turbidity, Hard – hardness, DO – dissolved oxygen, NO_3–nitrates, NO_2–nitrites, NH_4–ammonia, PO_4–phosphates, C_6H_5OH – phenol, SiO_2–silica, Fe-w – iron (in water). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Guimarães et al., 2009; Guimaraes et al., 2012). The *Barra* site presented the lower levels of nitrates and phosphates. The highest values of nitrites, ammonia, and phosphates, found at *Vagueira*, suggest a higher pollution by organic materials at this site relatively to the others. This was expected since this site is located in the Mira channel, which receives agriculture (fertilizers and pesticides) and animal farming effluents. These results highlight the importance of regular monitoring and intervention to improve water quality of these areas. In addition to water column, the sandy bottom is an important habitat for many fish species, including *P. microps*, and it provides suitable conditions for developing young and small fishes away from larger predators (Waligóra-Borek et al., 2005). Therefore, the sediment characterization is very important, especially when species living in close association with sediments, such as *P. microps*, are used as sentinel species. In fact, the type of sediment may influence fish distribution due to preferences for a particular kind of habitat (e.g. sandy, muddy), availability of food and other factors. Moreover, particle size and organic matter content are important factors regarding the bioavailability of several chemicals, including metals (Luo et al., 2011; Van Der Oost et al., 2003). In the present study, the higher mean concentrations of chlorophylls and phaeopigments were found at *Vagueira*, suggesting a higher primary productivity at this site, therefore in good agreement with the increased concentrations of nutrients in the water. Increased turbidity at this site may induce the resuspension of pollutants accumulated in the sediment. The concentrations of metals in sediments determined in the present study are in the range of those found by other authors in the Aveiro lagoon (Abreu et al., 2000; Fonseca et al., 2015; Martins et al., 2013; Monterroso et al., 2003b; Pereira et al., 1997, 2006; Serafim et al., 2013). Relatively to the other sites, *Vagueira* showed higher contamination by Al, Cr, Co and Ni, while *C. Bico* sediments were particularly contaminated with Pb, Zn and Hg. The concentrations observed for the *Vagueira* contrast with those observed by Fonseca et al. (2011). In addition, the results obtained for the *C. Bico* agreed with other works, who identified this area as being polluted by metals, in particular by Hg (Abreu et al., 2013; Fonseca et al., 2015; Monterroso et al., 2007; Nilin et al., 2012). Interestingly, the majority of metal concentrations in sediments decayed during Summer and Autumn periods in *Barra* and *Harbour*, probably reflecting lower industrial and shipping activity. Considering the sum of all the metals (Table A2 in Appendix A), the most contaminated sediments are those of *Vagueira*, followed by *C. Bico*, *Barra* and *Harbour*. This ranking is in good accordance with tidal residence time modelled by Lopes and Dias (2007). Significant statistical differences among seasons were also found for the majority of metals. The lagoon complex characteristics associated with the seasonal processes of suspension/transport/deposition/re-suspension can be considered for the redistribution of metal loads throughout the entire waterbody (Dias et al., 2001; Lopes and Dias, 2007; Pastorinho et al., 2012). The overall results reinforce the importance of regular and long-term sampling in coastal ecosystems with complex dynamics, as the Aveiro lagoon, contributing to improve the ecological risk assessment models.

4.2. Bioaccumulation of metals

In the case of metals, sediments may act as a “sink” that removes metals from the water column (Mota et al., 2005). Benthonic animals living in close association with the sediment, such as juvenile gobies that always stay close to the bottom (Hampel et al., 2003; Patzner et al., 2011) or feeding from benthonic preys, may be particularly exposed to metals and often these chemicals are found in their body. The concentrations observed in the selected species are in good accordance with corresponding concentrations that have been reported for several fish species from the Aveiro lagoon (Abreu et al., 2000; Eira et al., 2009; Lucas et al., 1986), including *P. microps* (Fonseca et al., 2011) and are in the range of concentrations found in *Pomatoschistus* spp. from other European estuaries. For example, Miramand et al. (1998) found concentrations of copper of 2.5 ± 1 μg/g (dry weight) in *P. microps* from the Seine estuary, while Geffen et al. (1998) reported Hg concentrations between 29.8 ± 18.29 and 43.93 ± 13.43 μg/g (wet weight) in *Pomatoschistus minutus*. Mean concentrations of Cr of 0.94 μg/g (dry wet weight) were observed by Caçador et al. (2012) in *P. microps* captured in the Seixal Bay, being the levels of Zn, Cu, Pb, Ni, Co and Cd

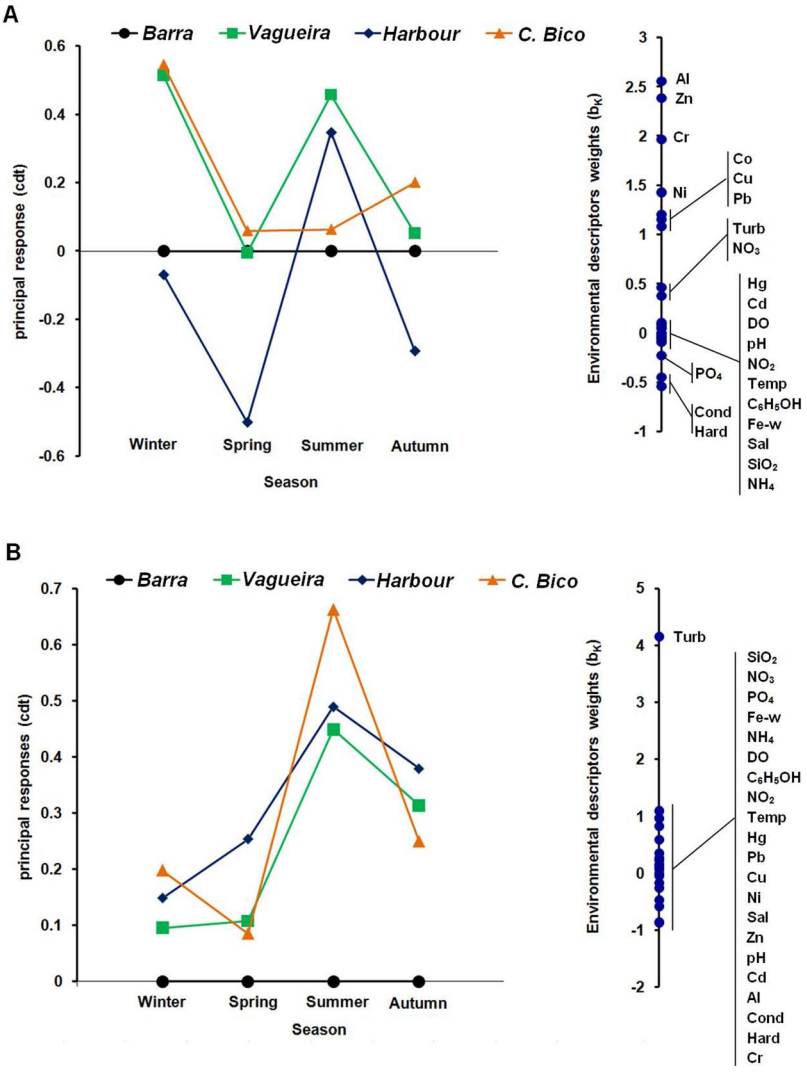

Fig. 3. First Principal Response Curves (PRC) resulting from the analysis of the environmental descriptors (water parameters and metals analysed in sediment), for the first axis (A) and the second axis (B). The lines represent the course of each sampling site levels in time. The descriptors weight (b_k) can be interpreted as the affinity of each described parameter with the Principal Response Curves.

considerably higher comparably to those observed in the present selected species.

The common goby was found to bioaccumulate Zn, Hg and Cu at all the sampling sites (Table 4); Ni at *Barra*, *Vagueira* and *C. Bico*; Cr at *Vagueira*, *Barra* and *Harbour*; and Co at *Harbour*. These results raises concern, considering their toxicity and the potential for bioaccumulation to higher trophic levels, including several fish consumed by humans (Van Ael et al., 2017). No bioaccumulation was found for Al despite the high concentrations of this metal found in sediment samples from all the sites, nor for Pb. The higher BAF values were found for Zn, mainly at *Harbour* area (20.96 ± 6.052). Despite the sediment concentrations of Al, Cr, Co and Ni were significantly higher at *Vagueira*, the concentrations of these metals in the *P. microps* were found to be similar or lower (Al) than those from the remaining sites. This suggests that the bioaccumulation of metals in *P. microps* was not only dependent directly from the sediment concentrations. It can be hypothesized the possibility that the *Vagueira* environmental conditions (e.g. organic and particulate matter and currents) may influence negatively the metal availability to the fish. These results reflect issues that deserve further investigation in relation to trophic implications and the importance of abiotic environment in these processes.

4.3. Condition indexes and biomarkers in fish

The FCF has been demonstrated to be a measure of the energy reserves of fish (Lloret et al., 2002; Mozsár et al., 2015) and can provide information on potential pollution impacts (Van Der Oost et al., 2003). In fact, healthy fish are expected to have higher FCF than animals living in polluted environments. However, this is not always the case. For example, in polluted environments where the food is abundant, exposed fish may accumulate a considerable amount of fat together with lipophilic toxicants that may decrease the reproductive success or even cause death if the fat is mobilised in a short period of time (Guimarães et al., 2009). In the present research, FCF revealed not to be a suitable parameter for contamination assessment, since no significant differences in this index were found among fish from distinct sites. Another commonly used index is the HSI that can be related to the accumulation of energy stores in the liver, to the accumulation of toxic substances in this organ and to exposure to chemicals causing liver injury (Al-Ghais, 2013; Guimarães et al., 2009). Here, the fish collected at *Harbour, Vagueira* and *C. Bico* showed significantly higher HSI levels relatively to those from the reference site, suggesting that fish may have been exposed for long periods of time to contaminants, inducing liver weight increase.

The fish collected at *Vagueira*, *Harbour* and *C. Bico* showed significantly inhibition of AChE activities suggesting exposure to anticholinesterase agents since the inhibition found (24%, 25%, 27%, respectively) is above 20%, the threshold usually considered as indicative of exposure to these agents (Ludke et al., 1975). These agents may be metals, but since known anti-cholinesterase agents of this group (e.g. Cu, Zn, Hg, Cr) were found also in fish from *Barra*, it is more likely that other chemicals (e.g. pesticides, PAHs, detergents) are causing the inhibition. Significantly inhibition of LDH activity was also observed in fish from *Vagueira*, *Harbour* and *C. Bico* relatively to those from *Barra*,

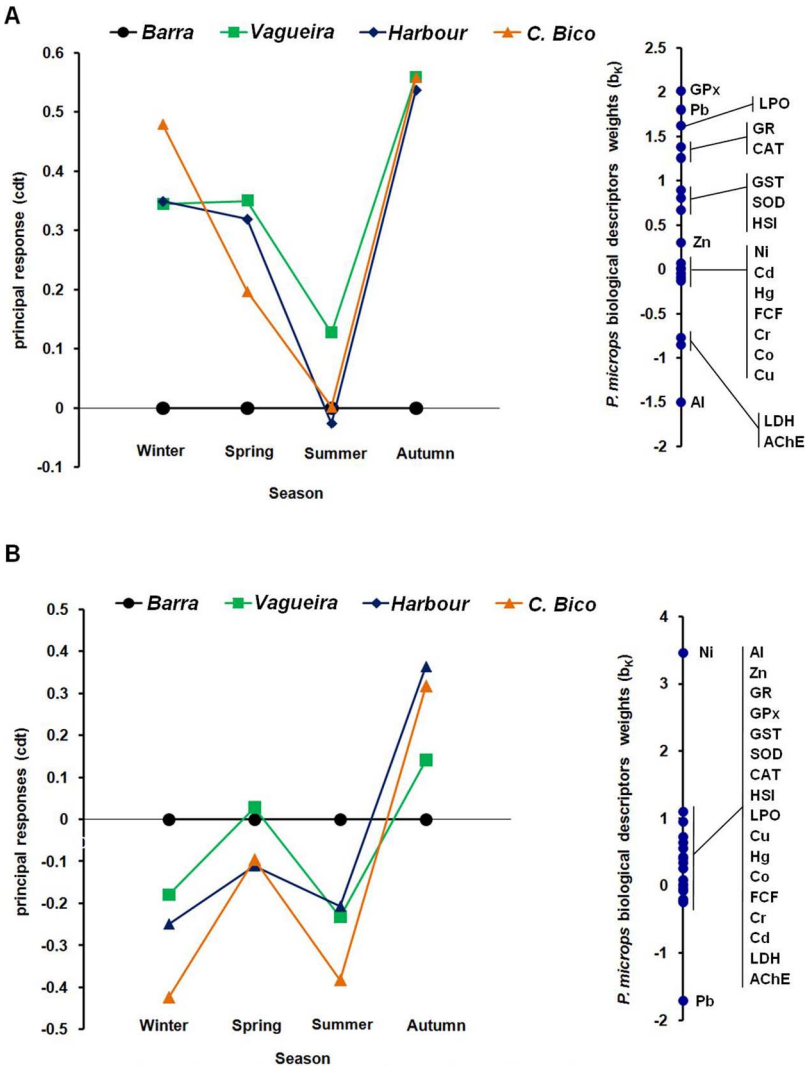

Fig. 4. PRC resulting from the analysis of the biological descriptors of *P. microps* (biomarkers, condition indexes and metals). (A) First axis; (B) Second axis. The lines represent the course of each sampling site levels in time. The descriptors weight (b_k) can be interpreted as the affinity of each described parameter with the Principal Response Curves.

suggesting that contamination is inducing changes in the pathways of cellular energy production. At contaminated sites, fish also showed increased activities of GST, suggesting exposure to xenobiotics with electrophilic centres (e.g. PAHs, some pesticides). In addition, increased activities of anti-oxidant enzymes were observed in the most contaminated sites, indicating exposure to oxidative agents and higher levels of LPO indicating oxidative damage (Vieira et al., 2009). The enhancement of GSTs, anti-oxidant enzymes and lipid peroxidation levels was previously reported for this species in laboratorial exposures to Cu an Hg (Vieira et al., 2009) and metal contaminated sites from the NW coast of Portugal (Guimaraes et al., 2012).

4.4. Integrated multivariate analysis of ecological and ecotoxicological parameters

In environmental monitoring studies it is important to consider the dependence of enzymatic biomarkers responses from the abiotic environment. In fact, seasonal changes in local levels of temperature, pH, salinity, nutrient and metals concentrations and fish behaviour, including feeding and reproductive habits, must be considered as determinant factors for the high observed variability in biomarkers responses (Guimaraes et al., 2012). In this context, an integrated analysis of obtained data may contribute for the translation of organism's responses to environmental stressors.

Concerning RDA analysis output, the first constrained axis (horizontal) was found to be strongly associated with a seasonality gradient, suggesting a variation of environmental conditions along the year. A group of environmental parameters including silica, iron, turbidity, pH, phenol and ammonia in the water, and Pb, Co, Cr, Zn and Al in sediments were associated with the autumn at *Vagueira*, *Harbour* and *C. Bico* (positive part of the axis). This association of some metals (Al, Pb and to a less extent Cr, Zn and Co) in sediments and the levels of turbidity and silica suggests their re-suspension from sediments after the first autumnal rains. It is also important to highlight the presence of iron within this group since the chemistry of aqueous iron has a major role in controlling the availability of many contaminants in re-suspended sediment (Jones-Lee and Lee, 2005). The second constrained axis (vertical) was found to be particularly influenced by contamination gradient since the reference site is separated from the contaminated sites, and no data, related with the *Barra* (circles), was found in the negative side of axis. Concerning the integrated analysis, the results of the vertical axis suggested that the levels of biomarkers are mainly affected by the contamination gradient, being separated in two groups: (1) AChE and LDH being associated with the reference, especially in the

autumn and (2) LPO, GST and antioxidant enzymes, associated with the negative part of the second axis, more related with the contaminated sites, indicating that the metals present in these areas increase the ROS levels in this fish species and the antioxidant system responds to an increase of environmental stress.

It is interesting to note the association between these parameters and Cu (both in fish and sediments) a metal known to cause lipid peroxidation, and the association of LPO and anti-oxidant enzymes with high levels of nutrients. These results are in good agreement with the findings of Ognjanović et al. (2008), indicating that high concentrations of nitrites and nitrates in the water may have been a direct result of oxidative stress and loading of LPO in fish. Fonseca et al. (2011) observed similar correlations between metal concentrations in sediments and anti-oxidant enzymes measured in the common goby captured in the same ecosystem. In the RDA it is also evident an opposite relation between two biomarkers (AChE and LDH) and metals in sediments, especially Zn, Co, Pb, Al, and Cr suggesting that the presence of these metals may have negative effects on neurotransmission and energy metabolism. It should be noted that RDA displays absolute comparisons, including several biological and environmental descriptors and covariates, which are positioned in function of each parameter weight. In this distribution, and despite the seasonal variability found, biomarkers were found to respond to a contamination gradient, separating the reference from the contaminated sites, especially during the autumn and summer.

The two groups of descriptors (biological and environmental) integrated in RDA were also analysed separately, in terms of relative comparisons, in a PRC analysis for both RDA axis (Figs. 3 and 4). The differences found in environmental descriptors (Fig. 3) between the reference and contaminated sites were mainly associated with Al, Zn and Cr concentrations in sediments, for the first axis. For the second axis the differences were found to be mainly associated with turbidity, reinforcing the importance of this parameter, influencing the metal concentrations in the coastal lagoon sediments. In relation to biological descriptors, the main differences between reference and remaining sites were associated with oxidative stress (GPx, LPO, GR and CAT), lipid peroxidation levels and Pb and Al in fish. In the PRC2 results, the observed differences were found to be mainly associated with Ni and Pb levels in *P. microps* body.

The overall results from integrated (RDA and PRC) analyses indicated that the AChE, LDH and the levels of LPO provided a clear distinction between reference and contaminated sites, following the results from other studies in the same coastal lagoon and from other NW Portuguese coastal ecosystems (Fonseca et al., 2011; Guimaraes et al., 2012). Thus, these enzymatic biomarkers may represent useful tools to assess epibenthic fish health status in these ecosystems (Guimaraes et al., 2012) and as an important component to be integrated in multivariate coastal ecosystem monitoring studies. In addition, the GST and the selected anti-oxidant enzymes revealed to be useful parameters to provide complementary information from the ecosystems health status. The characterization of water quality and the determination of important contaminants concentrations in both sediment and organisms proved to be determinant to assess possible toxic effects, pollutants accumulation and interpret biomarkers responses, being key parameters to be included in monitoring programs. Accordingly to the recent Intergovernmental Panel on Climate Change report (IPCC) (IPCC, 2014), the occurrence of heat waves, droughts and floods will tend to be more pronounced in several regions of the world, including the Southern European coastal ecosystems. This highlights the importance of regular and long-term integrated multivariate characterization of sensitive coastal ecosystems. The common goby is an important intermediary predator in costal ecosystems, controlling macro and meiofauna communities, being, also, an important prey for higher predators during its entire life cycle. Thus, the observed bioaccumulation of Hg raises concern, considering the toxicity of this metal and the potential of biomagnification (when in the organic form) to higher trophic levels, including several fish consumed by humans. This is an important issue that deserves further attention. The overall results also highlighted the importance of the selection of appropriate species for monitoring habitat health.

The integration of all parameters and methods discriminated locations with different contamination levels and associated health status of the selected fish with abiotic and metal pollution. This integrated approach can be used as an useful tool to improve the management of these ecosystems. Obtained results can, also, contribute for mathematical models development for conservation of living marine resources and for environmental management. This knowledge is essential to develop adaptive strategies to minimize losses of ecosystem services.

5. Conclusions

The present study proposed and validated a field monitoring approach, including ecological and ecotoxicological tools and multivariate statistics to integrate all the data. Furthermore, PRC analysis provided useful information regarding the discriminating power of parameters inside both biological and environmental descriptors groups, highlighting the most relevant parameters. Overall, the results of integrated analysis indicated that the selected battery of biomarkers, the concentrations of metals in both sediments and fish body and water quality variables were valuable parameters, discriminating different pollution scenarios and seasonal effects, also providing complementary information, therefore, being useful tools for environmental monitoring studies. On the contrary, the FCF proved not to be a good discriminatory parameter. The results also indicate that the selected fish can be considered a suitable species for use as bioindicator (sentinel species).

The present investigation represented a case-study, highlighting the use of enzymatic biomarkers as early warning indicators of ecosystems health and stressing the importance of using an integrated methodology through multivariate analysis of ecological and ecotoxicological parameters in biomonitoring studies, providing an important baseline and integrated information that may be used as scientific support for conservation and management and ecological models of estuarine and lagoon systems.

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Appendix A

Table A1
Mean values of the environmental data in water and sediments from seasonal sampling at the four selected sites at the Aveiro lagoon. The mean values, per year, for each site are shown in the grey column. Minimum and maximum (min-max) values are shown within brackets.

	Barra					Vagueira					Harbour					C. Bico				
Water	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean
Temperature (ºC)	12.1 (9.00 - 16.3)	20.9 (19.0 - 22.9)	27.6 (26.6 - 28.5)	18.3 (14.9 - 22.1)	19.7 (9.00 - 28.5)	12.9 (9.10 - 16.7)	21.4 (18.0 - 23.1)	27.6 (26.7 - 28.5)	18.5 (14.8 - 21.2)	20.1 (9.10 - 28.5)	12.8 (9.80 - 15.5)	20.7 (19.0 - 21.07)	26.0 (25.7 - 26.6)	17.6 (14.1 - 23.3)	19.3 (9.80 - 15.5)	12.2 (8.80 - 17.3)	21.9 (20.0 - 23.3)	27.0 (25.7 - 29.5)	18.0 (15.3 - 22.0)	19.8 (8.80 - 29.5)
Conductivity (m/S)	45.9 (40.1 - 50.2)	39.4 (22.6 - 48.8)	43.5 (35.9 - 51.1)	33.5 (29.8 - 38.8)	40.6 (22.6 - 51.1)	28.1 (16.8 - 39.4)	30.4 (24.5 - 37.3)	41.2 (33.2 - 49.1)	12.14 (9.37 - 19.2)	27.9 (9.37 - 49.1)	38.7 (29.8 - 48.4)	39.4 (32.8 - 45.9)	41.2 (36.0 - 51.6)	16.9 (12.4 - 28.9)	34.0 (12.4 - 28.9)	17.8 (15.7 - 19.8)	30.4 (6.35 - 43.5)	47.5 (45.3 - 51.3)	19.2 (11.2 - 31.8)	28.7 (6.35 - 51.3)
Salinity (‰)	18.2 (17.0 - 24.4)	18.1 (15.5 - 21.8)	22.7 (20.6 - 27.7)	20.0 (18.1 - 24.9)	19.8 (15.5 - 27.7)	14.2 (11.6 - 15.9)	17.9 (16.4 - 19.6)	22.3 (20.3 - 23.5)	16.93 (15.7 - 17.9)	17.8 (11.6 - 23.5)	16.9 (12.8 - 20.7)	18.0 (12.7 - 21.9)	20.3 (15.7 - 23.7)	15.1 (12.1 - 16.9)	17.6 (12.1 - 23.7)	13.4 (9.30 - 15.8)	15.4 (5.80 - 20.4)	19.8 (12.1 - 23.1)	16.2 (12.3 - 23.1)	16.2 (5.80 - 23.1)
pH	8.09 (8.04 - 8.16)	7.02 (5.80 - 7.95)	8.05 (7.52 - 8.46)	7.66 (7.09 - 8.19)	7.70 (5.80 - 8.46)	7.73 (7.04 - 8.35)	6.82 (5.95 - 7.91)	7.94 (7.42 - 8.36)	7.73 (7.50 - 8.07)	7.56 (5.95 - 8.36)	7.99 (7.75 - 8.18)	7.23 (6.24 - 7.92)	8.00 (7.28 - 8.43)	7.34 (6.74 - 8.45)	7.64 (6.24 - 8.45)	10.3 (7.18 - 15.7)	7.26 (5.91 - 7.95)	7.97 (7.23 - 8.37)	6.95 (6.36 - 7.80)	8.12 (5.91 - 15.7)
Dissolved O_2 (%)	91.10 (38.1 - 110)	81.4 (56.5 - 119)	59.7 (36.8 - 98.1)	78.1 (44.2 - 98.5)	77.6 (36.8 - 119)	80.7 (38.9 - 112)	64.3 (42.8 - 110)	62.5 (47.5 - 70.4)	90.9 (48.6 - 97.2)	74.6 (38.9 - 112)	57.3 (30.2 - 93.5)	68.1 (44.8 - 110)	48.9 (33.1 - 78.1)	79.7 (44.6 - 96.1)	63.5 (30.2- 110)	80.2 (45.7 - 101.5)	85.5 (48.6 - 106.8)	40.2 (34.9 - 99.8)	85.7 (40.9 - 71.6)	72.9 (34.9 - 111)
Nitrates (mg NO_3/L)	0.41 (0.22 - 0.58)	0.24 (0.05 - 0.34)	0.22 (0.20 - 0.24)	0.61 (0.30 - 0.90)	0.37 (0.05 - 0.90)	0.67 (0.44 - 1.06)	0.41 (0.36 - 0.50)	0.32 (0.26 - 0.38)	2.94 (0.48 - 4.45)	1.09 (0.26 - 4.45)	1.55 (1.02 - 2.60)	0.61 (0.44 - 0.74)	0.24 (0.23 - 0.24)	1.11 (0.68 - 1.94)	0.88 (0.23 - 2.60)	2.26 (0.90 - 3.80)	0.71 (0.60 - 0.86)	0.27 (0.24 - 0.32)	1.69 (0.54 - 3.10)	1.23 (0.24 - 3.80)
Nitrites (mg NO_2/L)	0.01 (0.00 - 0.01)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	0.07 (0.05 - 0.09)	0.02 (0.00 - 0.09)	0.06 (0.01 - 0.11)	0.00 (0.00 - 0.00)	0.01 (0.01 - 0.02)	0.17 (0.14 - 0.28)	0.06 (0.00 - 0.17)	0.30 (0.05 - 0.55)	0.02 (0.01 - 0.03)	0.02 (0.01 - 0.02)	0.05 (0.04 - 0.07)	0.10 (0.01 - 055)	0.03 (0.01 - 0.06)	0.04 (0.02 - 0.07)	0.04 (0.04 - 0.05)	0.10 (0.08 - 0.11)	0.05 (0.01 - 0.11)
Ammonia (mg NH_4/L)	0.28 (0.25 - 0.40)	0.21 (0.05 - 0.39)	0.48 (0.24 - 0.61)	0.86 (0.48 - 1.25)	0.46 (0.05 - 1.25)	0.46 (0.05 - 0.68)	0.76 (0.32 - 1.08)	0.37 (0.22 - 0.73)	0.87 (0.23 - 1.42)	0.61 (0.05 - 1.42)	0.47 (0.24 - 0.89)	0.69 (0.23 - 0.92)	0.47 (0.23 - 0.73)	0.73 (0.30 - 1.05)	0.59 (0.23 - 1.05)	0.31 (0.12 - 0.52)	0.52 (0.16 - 1.15)	0.56 (0.47 - 0.67)	0.74 (0.65 - 0.80)	0.53 (0.12 - 1.15)
Phosphates (mg PO_4/L)	0.12 (0.10 - 0.16)	0.25 (0.07 - 0.37)	0.05 (0.04 - 0.07)	0.20 (0.15 - 0.27)	0.16 (0.04 - 0.37)	0.21 (0.11 - 0.25)	0.28 (0.05 - 0.45)	0.16 (0.12 - 0.24)	0.92 (0.20 - 2.40)	0.39 (0.05 - 2.40)	0.80 (0.13 - 2.10)	0.86 (0.19 - 2.10)	0.06 (0.03 - 0.07)	0.32 (0.23 - 0.40)	0.51 (0.03 - 2.10)	0.38 (0.10 - 0.75)	0.17 (0.06 - 0.29)	0.15 (0.08 - 0.21)	1.15 (0.34 - 2.10)	0.46 (0.06 - 2.10)
Silica (mg Si/L)	1.03 (0.50 - 1.45)	1.10 (0.60 - 1.45)	0.27 (0.20 - 0.34)	2.63 (2.45 - 2.80)	1.26 (0.20 - 2.80)	1.07 (0.62 - 1.70)	1.90 (1.55 - 2.25)	1.06 (0.88 - 1.40)	2.50 (0.19 - 3.90)	1.63 (0.19 - 3.90)	1.92 (1.40 - 2.80)	1.03 (0.84 - 1.35)	0.30 (0.29 - 0.30)	3.55 (2.20 - 4.54)	1.70 (0.29 - 4.54)	2.15 (0.96 - 2.80)	1.07 (0.60 - 1.45)	1.09 (0.78 - 1.40)	2.50 (2.10 - 2.75)	1.70 (0.60 - 2.80)
Phenol (mg C_6H_5OH/L)	0.17 (0.10 - 0.28)	0.08 (0.06 - 0.10)	0.07 (0.00 - 0.14)	0.16 (0.06 - 0.22)	0.12 (0.00 - 0.28)	0.05 (0.01 - 0.10)	0.05 (0.03 - 0.10)	0.12 (0.07 - 0.17)	0.62 (0.28 - 1.70)	0.21 (0.01 - 1.70)	0.09 (0.07 - 0.11)	0.10 (0.05 - 0.16)	0.11 (0.08 - 0.14)	0.21 (0.11 - 0.30)	0.13 (0.05 - 0.30)	0.14 (0.09 - 0.20)	0.06 (0.05 - 0.09)	0.14 (0.11 - 0.17)	0.13 (0.10 - 0.17)	0.12 (0.05 - 0.20)
Iron (mg Fe/L)	0.17 (0.00 - 0.50)	0.00 (0.00- 0.00)	0.00 (0.00 - 0.00)	0.17 (0.00 - 0.30)	0.08 (0.00 - 0.50)	0.27 (0.00 - 0.75)	0.19 (0.00 - 0.55)	0.03 (0.00 - 0.05)	0.68 (0.35 - 1.15)	0.29 (0.00 - 1.15)	0.03 (0.00 - 0.05)	0.42 (0.00 - 0.90)	0.01 (0.00 - 0.01)	1.47 (0.15 - 2.40)	0.48 (0.00 - 2.40)	0.44 (0.00 - 1.30)	0.59 (0.01 - 0.90)	0.03 (0.01 - 0.05)	0.72 (0.45 - 1.20)	0.44 (0.00 - 1.30)
Hardness (mg $CaCO_3$ /L)	423 (330 - 510)	337 (210 - 550)	810 (710 - 830)	683 (630 - 730)	563 (210 - 830)	310 (190 - 390)	203 (110 - 390)	617 (530 - 690)	290 (250 - 330)	355 (110 - 690)	283 (130 - 390)	250 (130- 330)	457 (290 - 510)	607 (430 - 690)	399 (130 - 690)	250 (130 - 330)	197 (102-230)	510 (410 - 580)	337 (330 - 350)	323 (130 - 580)
Turbidity (FTU)	2.67 (0.00 - 6.00)	2.00 (0.00 - 4.00)	0.00 (0.00 -0.00)	5.67 (4.00 - 10.0)	2.58 (0.00 - 10.0)	3.00 (0.00 - 6.01)	3.00 (0.00 - 6.00)	4.00 (0.00 - 6.00)	11.1 (6.00 - 20.0)	5.28 (0.00 - 20.0)	3.00 (1.00 - 4.00)	4.00 (2.00 - 7.00)	6.00 (2.00 - 9.00)	23.0 (15.0 - 25.0)	9.00 (1.00 - 25.0)	2.33 (1.00 - 5.00)	2.67 (0.00 - 4.00)	10.67 (2.00 - 15.0)	10.7 (8.00 - 20.0)	6.58 (0.00 - 20.0)
Sediment																				
Chlorophyll *a* (mg/m^3)	0.105 (0.032 - 0.268)	0.246 (0.145 - 0.355)	0.176 (0.097 - 0.259)	0.128 (0.111 - 0.182)	0.164 (0.032 - 355)	0.355 (0.173 - 0.571)	0.226 (0.125 - 0.335)	0.217 (0.119 - 0.321)	0.177 (0.157 - 0.189)	0.244 (0.119 - 0.571)	0.256 (0.167 - 0.378)	0.212 (0.113 - 0.302)	0.221 (0.119 - 0.321)	0.089 (0.086 - 0.094)	0.195 (0.086 - 0.378)	0.180 (0.142 - 0.247)	0.162 (0.086 - 0.231)	0.062 (0.032 - 0.086)	0.070 (0.067 - 0.074)	0.119 (0.032 - 0.247)
Chlorophyll *b* (mg/m^3)	0.030 (0.001 - 0.109)	0.073 (0.023 - 0.101)	0.049 (0.038 - 0.064)	0.031 (0.027 - 0.037)	0.046 (0.001 - 0.109)	0.096 (0.015 - 0.204)	0.060 (0.023 - 0.096)	0.029 (0.013 - 0.055)	0.037 (0.033 - 0.044)	0.055 (0.012 - 0.204)	0.054 (0.032 - 0.082)	0.050 (0.029 - 0.075)	0.019 (0.014 - 0.019)	0.026 (0.021 - 0.028)	0.037 (0.014 - 0.082)	0.035 (0.016 - 0.062)	0.059 (0.035 - 0.079)	0.007 (0.006 - 0.009)	0.014 (0.011 - 0.015)	0.029 (0.006 - 0.079)
Chlorophyll *c* (mg/m^3)	0.013 (0.001 - 0.038)	0.135 (0.055 - 0.243)	0.136 (0.094 - 0.159)	0.060 (0.057 - 0.069)	0.086 (0.001 - 0.243)	0.288 (0.113 - 1.289)	0.101 (0.048 - 0.146)	0.152 (0.136 - 0.161)	0.059 (0.045 - 0.072)	0.150 (0.045 - 1.289)	0.131 (0.067 - 0.208)	0.108 (0.043 - 0.138)	0.149 (0.131 - 0.156)	0.056 (0.045 - 0.065)	0.111 (0.043 - 0.208)	0.069 (0.034 - 0.089)	0.056 (0.041 - 0.083)	0.109 (0.099 - 0.118)	0.024 (0.019 - 0.028)	0.065 (0.019 - 0.118)
Phaeopigments (mg/m^3)	1.922 (1.154 - 2.945)	7.128 (5.454 - 9.454)	4.031 (2.754 - 5.703)	1.178 (1.065 - 1.536)	3.564 (1.065 - 9.454)	8.523 (8.193 - 8.743)	5.492 (3.554 - 7.995)	1.628 (1.202 - 1.753)	2.426 (2.126 - 2.919)	4.517 (1.126 - 8.743)	3.218 (2.421 - 3.961)	3.942 (3.195 - 6.349)	0.628 (0.202 - 0.753)	0.867 (0.759 - 1.042)	2.164 (0.202 - 6.349)	1.897 (0.409 - 3.732)	3.322 (2.449 - 3.988)	2.496 (2.258 - 3.256)	2.004 (1.904 - 2.119)	2.430 (0.409 - 3.988)
Silt clay fraction (%)	22.94 (9.363 - 37.56)	14.20 (5.975 - 18.54)	6.37 (2.879 - 9.843)	7.68 (3.522 - 11.74)	12.797 (2.879 - 37.56)	11.12 (8.164 - 16.39)	7.73 (4.872 - 12.31)	13.97 (11.33 - 17.95)	9.82 (6.777 - 12.53)	10.66 (4.872 - 17.95)	8.12 (3.138 - 11.64)	5.64 (3.436 - 9.118)	4.77 (2.691 - 9.001)	6.01 (3.035 - 9.972)	6.13 (2.691 - 11.64)	11.43 (4.861 - 15.18)	24.05 (16.81 - 34.18)	4.52 (1.474 - 8.182)	7.22 (5.178 - 9.115)	11.81 (1.474 - 34.18)
Organic matter (%)	0.504 (0.372 - 0.687)	0.844 (0.069 - 1.441)	0.620 (0.362 - 0.989)	0.725 (0.551 - 0.993)	0.673 (0.069 - 1.441)	2.007 (1.224 - 2.851)	0.985 (0.677 - 1.792)	1.127 (0.758 - 1.481)	1.063 (0.797 - 1.241)	1.295 (0.677 - 2.851)	0.724 (0.173 - 0.963)	0.782 (0.141 - 1.429)	0.718 (0.535 - 1.101)	0.867 (0.653 - 1.075)	0.773 (0.141 - 1.429)	2.689 (1.554 - 4.706)	1.860 (1.409 - 2.177)	0.861 (0.683 - 1.127)	0.911 (0.671 - 1.109)	1.580 (0.671 - 4.706)

Table A2
Local and seasonal mean concentrations (μg/g) of the 9 selected metals measured in sediments, at the four selected sites. The grey column represents the mean values, per year, for each and total of metals (Σmetals). Values indicate the mean ± S.E.M., with exception of the total metal concentrations (μg/g) per season, shown at the bottom of the table. " < DL" – value below detection limit.

	Barra					Vagueira					Harbour					C. Bico				
	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean
Al	1067 ± 156.9	2840 ± 332.4	1028 ± 34.59	2602 ± 160.2	1885 ± 267.7	4972 ± 169.5	2508 ± 107.3	3388 ± 100.1	3667 ± 51.06	3634 ± 270.7	1024 ± 54.13	813.0 ± 71.33	1610 ± 75.71	1121 ± 108.3	1142 ± 94.45	4258 ± 99.16	2818 ± 658.8	672.3 ± 31769	3661 ± 210.3	2852 ± 435.9
Pb	0.993 ± 0.017	2.943 ± 0.214	2.110 ± 0.291	2.440 ± 0.291	2.122 ± 0.237	3.307 ± 0.073	2.347 ± 0.088	3.193 ± 0.082	3.230 ± 0.334	3.019 ± 0.414	2.137 ± 0.288	1.467 ± 0.126	3.210 ± 0.187	1.783 ± 0.083	2.149 ± 0.213	4.753 ± 0.143	3.903 ± 0.914	1.863 ± 0.234	4.250 ± 0.091	3.693 ± 0.383
Cd	< DL	< DL	< DL	< DL	< DL	0.210 ± 0.200	< DL	< DL	< DL	0.052 ± 0.051	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
Cr	2.190 ± 0.238	3.730 ± 0.520	0.787 ± 0.034	2.433 ± 0.237	2.285 ± 0.341	6.523 ± 0.301	3.290 ± 0.115	3.800 ± 0.175	1.773 ± 0.306	3.847 ± 0.527	1.643 ± 0.082	0.196 ± 0.188	1.397 ± 0.131	0.883 ± 0.191	1.029 ± 0.179	5.113 ± 0.199	2.837 ± 0.794	< DL	4.007 ± 0.351	2.990 ± 0.604
Co	0.341 ± 0.168	0.320 ± 0.311	< DL	1.011 ± 0.070	0.416 ± 0.133	2.150 ± 0.037	0.807 ± 0.014	1.197 ± 0.066	0.860 ± 0.060	1.253 ± 0.163	0.333 ± 0.013	< DL	0.217 ± 0.181	0.263 ± 0.228	0.190 ± 0.071	1.697 ± 0.046	1.120 ± 0.372	< DL	1.083 ± 0.352	0.883 ± 0.214
Cu	3.547 ± 0.525	2.900 ± 0.741	< DL	3.060 ± 0.693	2.378 ± 0.485	6.123 ± 0.532	2.260 ± 0.776	0.477 ± 0.441	1.877 ± 0.590	2.677 ± 0.683	0.797 ± 0.054	1.727 ± 0.276	2.657 ± 0.183	1.453 ± 0.344	1.658 ± 0.226	4.680 ± 0.142	3.197 ± 0.841	1.223 ± 1.188	3.927 ± 0.302	3.249 ± 0.504
Ni	2.330 ± 0.401	2.747 ± 0.598	0.233 ± 0.198	2.530 ± 0.339	1.953 ± 0.354	4.953 ± 0.076	3.683 ± 0.943	1.043 ± 0.128	1.993 ± 0.235	2.918 ± 0.501	1.200 ± 0.055	< DL	2.230 ± 0.257	1.340 ± 0.127	1.194 ± 0.246	4.277 ± 0.179	2.167 ± 0.462	1.697 ± 0.72	2.207 ± 0.191	2.587 ± 0.321
Zn	8.800 ± 0.812	14.77 ± 1.722	6.027 ± 0.137	17.50 ± 1.126	11.77 ± 1.457	23.43 ± 1.304	12.83 ± 1.047	14.23 ± 0.887	15.87 ± 0.841	16.59 ± 1.131	5.737 ± 0.438	4.193 ± 0.712	11.33 ± 0.504	5.733 ± 1.053	6.749 ± 0.875	38.53 ± 1.125	24.27 ± 4.299	7.10 ± 0.026	30.20 ± 1.873	25.03 ± 3.623
Hg	0.110 ± 0.026	0.070 ± 0.005	< DL	0.060 0.001	0.061 ± 0.012	0.183 ± 0.038	0.053 ± 0.003	0.057 ± 0.018	0.060 ± 0.004	0.085 ± 0.019	0.187 ± 0.056	0.067 ± 0.006	0.063 ± 0.021	< DL	0.077 ± 0.024	0.280 ± 0.033	0.157 ± 0.028	0.070 ± 0.015	0.120 ± 0.05	0.157 ± 0.025
Σmetals	1086	2867	1037	2631	1905 ± 490.1	5019	2533	3412	3692	3664 ± 515.3	1036	820.6	1631	1132	1155 ± 172.1	4317	2855	684.3	3707	2891 ± 794.4

Table A3
Local and seasonal mean concentrations (μg/g) of the nine selected metals measured in *P. microps*, collected at the four sites of Aveiro lagoon. The grey column indicates the mean values, per year, for each and total of metals (Σmetals). Values indicate the mean ± S.E.M., with exception of the total metal concentrations (μg/g) per season, shown at the bottom of the table. " < DL" – value below detection limit.

	Barra					Vagueira					Harbour					C. Bico				
	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean	WINTER	SPRING	SUMMER	AUTUMN	Annual mean
Al	636.3 ± 73.45	248.7 ± 31.92	74.50 ± 3.879	72.70 ± 4.611	258.1 ± 71.37	243.0 ± 36.11	239.3 ± 32.63	82.20 ± 22.47	45.10 ± 4.172	152.4 ± 29.38	334.1 ± 79.41	139.9 ± 26.12	74.70 ± 13.65	19.17 ± 2.338	141.9 ± 40.12	351.0 ± 15.01	385.0 ± 24.82	151.3 ± 13.54	26.50 ± 7.703	228.5 ± 44.82
Pb	< DL	0.460 ± 0.451	6.223 ± 1.571	< DL	1.673 ± 0.867	< DL	1.893 ± 0.453	3.027 ± 1.617	3.513 ± 2.118	2.102 ± 0.714	< DL	< DL	< DL	3.995 ± 2.018	1.003 ± 0.675	2.630 ± 0.902	< DL	1.273 ± 1.238	3.827 ± 1.903	1.704 ± 0.665
Cd	< DL	< DL	0.320 ± 0.026	< DL	0.081 ± 0.080	< DL	0.180 ± 0.059	0.343 ± 0.054	< DL	0.129 ± 0.081	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
Cr	0.303 ± 0.291	0.720 ± 0.242	2.530 ± 0.049	2.093 ± 0.342	1.350 ± 0.313	0.880 ± 0.291	0.643 ± 0.052	2.797 ± 0.518	1.700 ± 0.552	1.359 ± 0.342	0.270 ± 0.261	0.850 ± 0.283	2.773 ± 0.228	2.020 ± 0.209	1.407 ± 0.328	0.197 ± 0.188	0.563 ± 0.074	2.487 ± 0.127	1.947 ± 0.371	1.298 ± 0.297
Co	< DL	< DL	1.167 ± 0.645	1.970 ± 0.323	0.783 ± 0.293	< DL	< DL	1.343 ± 0.551	1.687 ± 0.875	0.756 ± 0.318	< DL	< DL	1.077 ± 0.082	2.022 ± 0.205	0.777 ± 0.257	< DL	< DL	1.047 ± 0.605	1.623 ± 0.291	0.666 ± 0.253
Cu	7.187 ± 0.280	9.140 ± 3.480	1.470 ± 0.872	4.003 ± 0.381	5.446 ± 1.176	9.347 ± 2.639	7.950 ± 0.228	2.957 ± 0.433	4.513 ± 0.735	6.192 ± 0.974	4.910 ± 0.275	8.667 ± 0.816	5.813 ± 0.916	4.210 ± 0.603	5.901 ± 0.591	5.287 ± 0.466	4.717 ± 0.768	9.700 ± 4.266	4.193 ± 0.157	5.974 ± 1.139
Ni	< DL	2.413 ± 0.973	1.883 ± 0.262	4.990 ± 1.179	2.323 ± 0.630	2.145 ± 0.854	1.363 ± 0.261	2.930 ± 0.608	1.743 ± 0.141	1.867 ± 0.301	3.035 ± 1.032	2.353 ± 0.443	0.923 ± 0.452	0.547 ± 0.511	1.451 ± 0.362	9.567 ± 3.521	1.590 ± 0.034	3.760 ± 0.490	0.820 ± 0.785	3.927 ± 1.293
Zn	89.03 ± 2.747	111.6 ± 13.44	60.37 ± 0.895	75.37 ± 2.351	84.10 ± 6.415	110.9 ± 14.81	147.3 ± 5.547	86.87 ± 6.570	75.00 ± 0.404	105.0 ± 9.099	167.0 ± 8.66	147.0 ± 4.582	105.8 ± 8.565	59.37 ± 4.941	119.8 ± 12.79	138.0 ± 4.041	138.7 ± 1.333	91.03 ± 3.071	65.17 ± 6.688	108.2 ± 9.588
Hg	0.437 ± 0.067	0.313 ± 0.0617	0.177 ± 0.003	0.160 ± 0.028	0.272 ± 0.039	0.480 ± 0.068	0.263 ± 0.053	0.147 ± 0.006	0.147 ± 0.008	0.259 ± 0.45	0.347 ± 0.023	0.240 ± 0.028	0.201 ± 0.020	0.143 ± 0.031	0.233 ± 0.025	0.550 ± 0.030	0.423 ± 0.056	0.313 ± 0.143	0.307 ± 0.085	0.398 ± 0.048
Σmetals	733.3	373.3	148.6	161.3	354.1 ± 136.5	366.8	399.0	182.6	133.4	270.1 ± 66.01	509.6	299.0	191.3	91.5	272.5 ± 89.57	507.2	531.0	260.9	104.4	350.6 ± 102.4

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